In search of cryptic species in two sawfly species with high mitochondrial DNA divergence

Master of Science Thesis in Ecology and Evolution

Kristian Brysting Kristiansen

Natural History Museum

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http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo

Forord

Jeg vil starte med å takke mine tre veiledere, Arild Johnsen, Geir Sørli og Ole Lønnve. Dere har gitt meg en spennende oppgave, blandet med litt felt, morfologi, labarbeid og analyser. Jeg vil takke for alle tilbakemeldinger i innspurten. Videre vil jeg takke Silje og Gunnhild og alle andre som gav meg hjelp og opplæring på labben.

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Abstract

A preliminary DNA barcoding project at the Natural History Museum, University of Oslo, revealed intraspecific variation in the mitochondrial gene cytochrome c oxidase subunit I (COI) within two sawfly species, *Pachyprotasis rapae* Linnaeus, 1767 and *Athalia circularis* Klug, 1815. The aim of this study was to investigate if the two different haplogroups discovered within each species represent cryptic species. To accomplish this, a larger sample of individuals was sequenced for both mitochondrial DNA (mtDNA; COI) and nuclear DNA (nDNA; CAD-gene). The general habitus (colouration) and parts of the female genitalia (the saw) were investigated to search for differences that might correspond with intraspecific genetic differences. Based on analyses of the mtDNA, each species was divided in two haplogroups. However, these haplogroups were not supported by the nDNA analyses. Even though high genetic variation was found among the analysed *P. rapae* samples (COI), analysis of the CAD-gene did not reveal any clear grouping. For *A. circularis*, analysis of the CAD-gene resulted in one monophyletic group and almost no genetic differences among the investigated specimens. There was no genetic variation (COI) between local populations of *P. rapae.* Populations of *A. circularis* showed significant genetic variation between several local populations; however, this result may be due to sampling bias and small sampling sizes. There was no difference in colouration or in the outline of the saw between the haplogroups in any of the species, but for both species, there were significant colour differences between the sexes.

Based on these results, neither *P. rapae* nor *A. circularis* show signs of harbouring cryptic species. *P. rapae* showed the highest genetic difference between mtDNA haplogroups (4 %) and a variation in the nDNA, which could be a sign of secondary contact after geographical separation. This result shows that the high divergence in mtDNA is not synonymous with cryptic species and that DNA barcoding should be supported by other genetic evidence and morphology when used for the purpose of species discovery. However, DNA barcoding is still useful for separating between the studied species and closely related species, as they were both monophyletic with respect to their out-groups.

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1 Introduction

The number of described species on earth today is believed to be around 1.2-1.5 million (Mora et al. 2011, Costello et al. 2013). However, the total number of existing species is much higher and could range from 3 to 100 million (May 2010), with recent estimates ranging from 5 to 8 million species (Mora et al. 2011, Costello et al. 2013). Due to the high number of unknown species and the shortage of taxonomists to deal with this (the taxonomic impediment), Hebert et al. (2003a) introduced the idea of DNA barcoding.

A DNA barcode is a standardized genetic sequence that shares unique features among members of a species. DNA barcoding has two main goals; the first is to make an identification library of all known species, and the second is screening of unknown biodiversity with the goal of identifying new species (Hebert et al. 2003a). Within the metazoan kingdom, the most commonly used DNA barcode marker is the mitochondrial gene cytochrome c oxidase subunit I (COI) (Ratnasingham and Hebert 2007). This gene is able to discriminate closely related metazoan species in most taxa (Hebert et al. 2003b, Ratnasingham and Hebert 2007). The mitochondrial DNA (mtDNA) is a circular closed molecule (Boyce et al. 1989, Boore 1999), which is maternally inherited, lacks recombination and is haploid with only one allele of each gene (Dawid and Blackler 1972, Wilson et al. 1985, Avise et al. 1987). MtDNA has 5-10 times higher mutation rate than nuclear DNA (nDNA). This is due to several factors such as; the lack of repair enzymes, the lack of histone binding, a corrosive oxygen-rich environment and that it does not produce proteins directly involved in its own replication, transcription or translation (Brown et al. 1979, Wilson et al. 1985, Avise 1986, Avise 2009). These properties make mtDNA highly suitable as a phylogenetic and phylogeographic marker in general (Avise 2000), and DNA barcoding marker in particular (Hebert et al. 2003b). The use of mtDNA as a molecular marker has, however, also been criticized (Moritz and Cicero 2004, Rubinoff et al. 2006, Elias et al. 2007, Whitworth et al. 2007, Song et al. 2008, Galtier et al. 2009). For instance, Galtier et al. (2009) argue and provide evidence that the basic assumptions about mtDNA are not always met; it is not always strictly maternally inherited (paternal leakage), it can be affected by recombination and positive selection, and has an erratic evolutionary rate.

Barcoding can be used to discover cryptic species. Cryptic species are species that morphologically look the same, but are genetically so different that they can be considered

separate species (Hebert et al. 2004, Barrett and Hebert 2005, Hajibabaei et al. 2006, Pfenninger and Schwenk 2007, Rasmussen et al. 2009, Steinke et al. 2009). For example, Hebert et al. (2004) discovered at least ten different species within what had previously been considered a single butterfly species, *Astraptes fulgerator* Walch, 1775, using barcoding (but see Brower 2006).

High genetic variation within the COI gene does not necessarily reflect the existence of cryptic species, as there are several examples of deep intraspecific divergences in mtDNA, even in sympatry (Chang et al. 2007, Avtzis et al. 2008, Hogner et al. 2012, Kvie et al. 2012). There are several alternative explanations for such divergences. First, intraspecific genetic variation may result from nuclear mitochondrial pseudogenes (numts). Numts are non-functional genes that have been transferred from the mtDNA into the nDNA. They can easily be coamplified and can be detected from the occurrence of double peaks and stop codons in sequences (Bensasson et al. 2001, Song et al. 2008). If undetected, numts could lead to an artificial increase in the number of unique species found using DNA barcoding (Song et al. 2008). Second, large intraspecific genetic variation could be caused by introgression via hybridization of closely related species (Coyne and Orr 2004, Linnen and Farrell 2007, Linnen and Farrell 2008, Zakharov et al. 2009, Nicholls et al. 2012). Third, lineage sorting can affect isolated populations of a species. Different genes are affected by different rates of lineage sorting, primarily driven by genetic drift and dependent on population size (Neigel and Avise 1986). Within each population the mtDNA will have a higher rate of mutation than the nDNA, which can lead to populations having different haplotypes of mtDNA and non-divergent nDNA simultaneously (Maddison 1997, Freeland 2005). When no reproductive barriers evolve during separation, there could be secondary contact when previously isolated populations come into contact. This could lead to the presence of different haplotypes of mtDNA in a population (Hewitt 1996, Knowles 2000, Chang et al. 2007, Zakharov et al. 2009). Finally, it could be caused by different strains of *Wolbachia* bacteria, which are cytoplasmic maternally inherited endosymbionts. *Wolbachia* affects the mating pattern in the host, with effects including parthenogenesis, reproductive incompatibility, feminization of genetic males and male killing (Werren et al. 1995, Werren and Windsor 2000, Wernegreen 2002, Coyne and Orr 2004, Hurst and Jiggins 2005, Smith et al. 2012). Hilgenboecker et al. (2008) estimated that at least 20 % of all insect species are infected with *Wolbachia.* This multitude of possible explanations for high intraspecific genetic variation within COI shows

that information from more than one molecular marker is often needed in order to delimit species (Elias et al. 2007, Galtier et al. 2009, Damm et al. 2010, Dupuis et al. 2012).

A gene tree is a phylogenetic tree that shows the evolutionary history of a single gene. It is important to remember that this differs from a species tree, which shows the evolutionary history of a group of species (Doyle 1992, Nichols 2001, Edwards 2009). A species tree must always be estimated from gene trees but the two are not necessarily the same (Doyle 1992). Incongruence between gene trees and species trees can arise from linage sorting, introgression via hybridization, horizontal gene transfer, gene duplication and gene extinction (Maddison 1997). Sequencing of a single gene, e.g. the standard gene for DNA barcoding COI (Ratnasingham and Hebert 2007), can thus be misleading with regard to true species relationships and should be interpreted carefully.

Symphyta is a suborder within the order Hymenoptera consisting of eight superfamilies and 25 families worldwide (Taeger et al. 2010). Ten of these families are found in Norway, the largest of which is Tenthredinidae, with about 300 species. However, it is believed that up to 700 species of Tenthredinidae can be found in our fauna (Ottesen 1993). This thesis is based on the preliminary results of a DNA barcoding project on Norwegian Symphyta, performed at the Natural History Museum, University of Oslo (NHM Oslo), which identified species worthy of further study (Lønnve, Lifjeld and Johnsen, unpublished). In this thesis I will focus on two of these species, both of which are members of the family Tenthredinidae: *Pachyprotasis rapae* Linnaeus, 1767 and *Athalia circularis* Klug, 1815.

Pachyprotasis Hartig, 1837 is a genus, which is very species rich in Asia, with about 200 species (Saini 2007). However, only five known species are found in Europe, *P. antennata* Klug, 1817, *P. nigronotata* Kriechbaumer, 1874*, P. rapae, P. simulans* Klug, 1817 and *P. variegata* Fallén, 1808 (Taeger et al. 2006). Four of these species are found in Norway, *P. antennata*, *P. rapae, P. simulans* and *P. variegata* (Taeger et al. 2006). All the Norwegian species except *P. simulans* were included in the preliminary barcoding project (NHM Oslo), which divided *P. rapae* into two different haplogroups with an almost 4 % genetic difference (based on seven individuals; four in one group and three in the other). Within *P. rapae,* individuals with different colouration on the thorax and the abdomen have been registered (Lønnve, personal comment) and the species is associated with a large range of host plants (Liston 1997, Taeger et al. 1998). *P. rapae* has a flight period in Norway from the end of May

to the end of June (Benson 1958), which can be delayed in elevated areas (Lønnve, personal comment).

In Norway, there are six known species of *Athalia* Leach, 1817, *A. circularis, A. cordata* Lepeletier, 1823*, A. liberta* Klug 1815, *A. lugens* Klug, 1815*, A. rosea* Linnaeus, 1758 and *A. scutellariae* Cameron, 1880 (Taeger et al. 2006). All species except *A. scutellariae* were included in the preliminary barcoding project (NHM Oslo), which divided *A. circularis* in two different haplogroups with an almost 2 % genetic difference (based on 10 individuals; seven in one group and three in the other). Mol (2009) recently elevated *A. longifoliae* Kontuniemi, 1951 from subspecies of *A. circularis* to a separate species. *A. circularis* also shows colour variation on the thorax, ranging from black to yellow (Benson 1962, Mol 2009). *A. circularis* has been reported with different host plant families (Asteraceae, Brassicaceae, Lamiaceae and Plantaginaceae) (Benson 1962, Taeger et al. 1998). Recent work showed that larger parts of the *Athalia* genus have been through a host plant shift, from Lamiales to Brassicaceae (Opitz et al. 2012), but that *A. circularis* only had successful development on the plant family Plantaginaceae (in Lamiales). *A. circularis* has a flight period from mid-June until mid/end of August.

The aim of this thesis is to investigate intraspecific variation in the two sawfly species, *P. rapae* and *A. circularis*, using DNA barcoding and morphological examination. This will be done by analysing a larger sample size of each species for intraspecific variation within COI, to test if the results from the preliminary analyses can be corroborated. Furthermore, the hypothesis that divergent lineages (haplogroups) are cryptic species will be tested by searching for corresponding patterns of intraspecific genetic variation in nDNA, using a part of the CAD-gene. Since both species vary in colouration, it will be examined whether colour differences correspond with the haplogroups, local populations and/or are sex dependent. Finally, differences in the female genitalia, the saw, will be investigated, as this structure has previously been used to distinguish between closely related Symphyta species (Heidemaa 2004, Heidemaa et al. 2004, Prous et al. 2011).

2 Materials and methods

2.1 Specimens

A total of 171 specimens were included (Appendix 1, Table 1-A), provided from either private collections, from BioFokus or from the collections at NHM Oslo. These specimens (*P. rape* n=70, *A. circularis* n=77, out-group species n=24) were collected in Norway (n=165), Sweden (n=5) and Scotland (n=1). Most were collected by a Malaise trap (Malaise 1937) and stored on ethanol (60-96 %) at -4 °C or lower. Other specimens were dried and pinned.

Additional sequences of COI were obtained from an unpublished Norwegian Barcode of Life (NorBOL) project at the NHM Oslo (Lønnve, Lifjeld and Johnsen, unpublished): *P. antennata* (n=2), *P. rapae* (n=5), *P. variegata* (n=4), *A. circularis* (n=9), *A. cordata* (n=4), *A. liberta* (n=4), *A. lungens* (n=5) and *A. rosae* (n=5).

2.2 DNA extraction

Two DNA extraction methods were used: E-Z 96® Tissue DNA Kit (Omega Bio-Tek, Norcross, GA, USA), a 96-well extraction method, and E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek), single tube extraction method.

One leg from each specimen was removed, placed in separate wells or tubes, and then dried at room temperature to get rid of excessive ethanol. After drying, 200µl TL buffer was added and the leg was crushed using a plastic stick. Then 25µl OB Protease was added into each well/tube followed by vortexing. The lysis reaction proceeded overnight. The rest of the extraction protocol followed the provided manual of the kit, resulting in a total of 200µl extraction volume for each specimen.

2.3 DNA amplification

Two different DNA regions were amplified, COI and a part of the carbamoylphosphate synthetase (CPS) part of the CAD-gene. Internal transcribed spacer 2 (ITS2) and *Wolbachia* outer surface protein gene (WSP) were also attempted amplified, but gave no product following the protocols from Kvie et al. (2012).

A 710 base pair (bp) fragment of COI was amplified by the forward primer LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and the reverse primer HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). The amplification used the following PCR profile: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min and a final extension step at 72 °C for 7 min.

A 1073 bp fragment of CPS of CAD was amplified by the forward primer 54F (5′-GTN GTN TTY CAR ACN GGN ATG GT-3′) and the reverse primer 405R (5′-GCN GTR TGY TCN GGR TGR AAY TG′) (Moulton and Wiegmann 2004). The amplification used the following PCR profile: initial denaturation at 94 °C for 3 min, followed by five cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 1.5 min, five cycles of 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 1.5 min, 35 cycles of 94 °C for 30 sec, 45 °C for 30 sec and 72 °C for 1.5 min and a final extension step at 72 °C for 7 min.

The PCR reactions were prepared in volumes of 10, 12.5, 15 or 25µl. Each reaction had a mixture of the following reagents and final concentrations: 1x PCR Rxn Buffer (Invitrogen, Carlsbad, CA, USA), 1.5 mM $MgCl_2$ (Invitrogen), 0.8 mM dNTP (AB, Warrington, UK), 0.2 µM of each primer, 1 U Platinum®Taq DNA polymerase (Invitrogen) and dH_2O to make the concentrations and PCR volume. Positive control was used when available and negative control (dH_2O) was included in all PCR setups. All PCR products were tested on a 1 % agarose gel using GelRed™ nucleic acid gel stain (Biotium, Hayward, CA, USA). Gels were loaded with 2.5µl PCR product and 2.5µl loading buffer and run at 85 V for 25 min. PCR products with low intensity or no band on the gel were amplified again with increased $MgCl_2$ and polymerase concentrations. PCR products were stored in a freezer, or a fridge for short term storage.

2.3.1 Sequencing

PCR reactions were cleaned using 0.4µl 10x diluted illustra™ ExoStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) per µl PCR product. To deactivate the enzymes, the mix was incubated at 37 °C for 45 min, followed by 80 °C for 15 min.

Sequencing was done by StarSEQ® (Mainz, Germany). The samples were shipped in 96-well plates. Each well contained 6µl of the cleaned sample and 1µl of either the forward or the

reverse primer. All PCR products were sequenced both ways. The plates were covered with Adhesive Sealing Sheets (Thermo Scientific, UK) and shipped with FedEx.

2.4 Sequence analyses

The analyses of COI included 44 sequences of *Pachyprotasis* (38 specimens of *P. rapae* and two out-group taxa: *P. antennata* (n =2) and *P. variegata* (n=4)), and 91 sequences of *Athalia* (74 specimens of *A. circularis* and four out-group taxa: *A. cordata* (n=5), *A. liberta* (n=4), *A. lungens* (n=5) and *A. rosae* (n=5)). The analyses of CAD included 15 sequences of *Pachyprotasis* (13 specimens of *P. rapae* and one out-group taxon: *P. variegata* (n=2)) and 15 sequences of *Athalia* (12 specimens of *A. circularis* and one out-group taxon: *A. cordata* (n=3)). The sequences were edited in CodonCoder Aligner version 3.7.1 (CodonCode Corp., Dedham, MA, USA). Sequences were cut using the maximized region option with an error rate below 0.05. Sequences shorter than 500 bp after the cut were removed. Consensus sequences of forward and reverse sequences were made using *assemble in the group* option. All consensus sequences were checked manually and edited when needed. The alignment of sequences was done in MEGA version 5 (Tamura et al. 2011) with the standard settings in Muscle (Edgar 2004).

The *Model test* option in MEGA was used to find the best nucleotide substitution model. The COI analyses of *Pachyprotasis* were done with the Tamura 3-parameter model. The CAD analyses of *Pachyprotasis* were done with the Kimura 2-parameter model. The COI analyses of *Athalia* were done with the Tamura-Nei model. The CAD analyses of *Athalia* were done with the Jukes-Cantor model. Neighbour-joining and Maximum Likelihood analyses were done in MEGA. All analyses were done using these options: *gamma distribution with five discrete categories*, *complete deletion* and *all codon sites included*. The *All substitutions* option was used in the Neighbour-joining analyses and the *Nearest-Neighbour-Interchange* option was used for the Maximum Likelihood analyses. The genetic distances between haplogroups (and between haplogroups and out-groups) were measured with the *pairwise distance* option in MEGA with same nucleotide substitution model used in phylogenetic analyses.

DnaSP (Librado and Rozas 2009) was used to find polymorphic details about the COI and CAD sequences.

Analyses of Molecular Variance (AMOVA) based on COI sequences were done in Arlequin (Excoffier and Lischer 2010) to investigate genetic structure among and within local populations. Pairwise F_{ST} values between populations were also estimated. Populations were based on the European invertebrate survey (EIS)-system (Figure 1), 32 specimens of *P. rapae* and 71 specimens of *A. circularis* from Norway (all with COI sequences) were divided into local populations. *Pachyprotasis* were divided into four populations and *Athalia* were divided into six populations (Table 1). List of specimens in the different populations can be found in Appendix 1 (Table 2-A).

Table 1. Local populations of *Pachyprotasis rapae* and *Athalia circularis* used in the AMOVA. Definition of local populations was based on the European invertebrate survey (EIS)-system.

Population	n	EIS square(s)
Pachyprotasis rapae		
1	11	36
2	11	19,27,28,29
3	6	45,53
4	4	37,38
Athalia circularis		
1	17	11
2	14	20
3	11	28
4	13	29
5	11	27,34
6	5	36

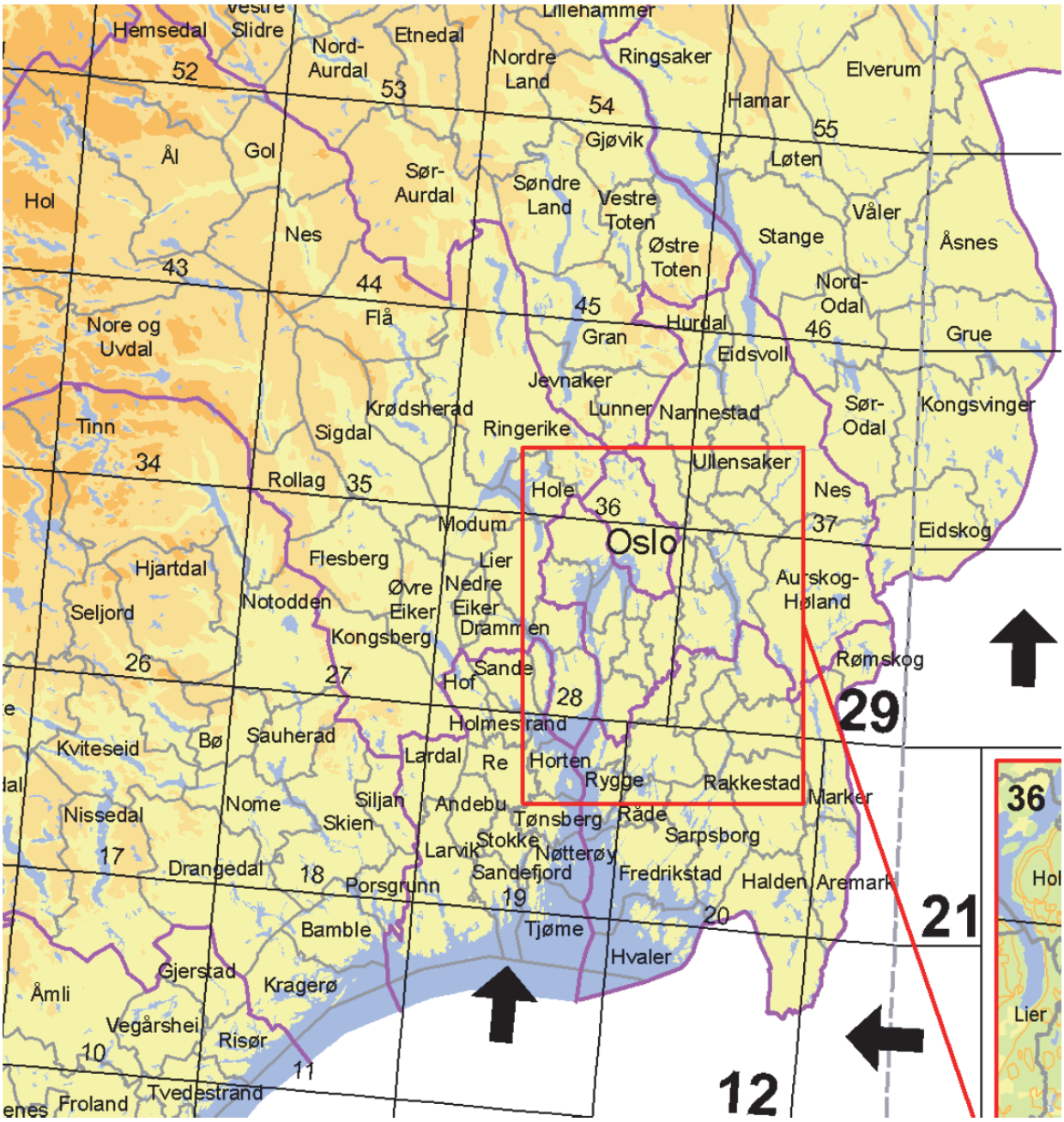

Figure 1. Part of the European invertebrate survey (EIS)-system of Norway (Endrestøl 2005).

2.5 Morphology

Body morphology was scored with a stereo microscope (x16, Wild M3B, Heerbrugg, Switzerland). Genitalia were prepared with a stereo microscope (Wild M8) and with an optical microscope (Leica DMLB, Wetzlar, Germany), and pictures of the genitalia were taken with an optical microscope (Leica DM6000B). The morphology analyses were done on 70 specimens of *P. rapae* and 77 specimens of *A. circularis.* All specimens from the DNA analyses were included in the morphology analyses.

2.5.1 *Pachyprotasis rapae*

Differences in colour between individuals of *P. rapae* have previously been observed (Lønnve, personal comment). Three different colour characters were chosen (Figure 2), which were all scored with three character states (Table 2). The first character concerns the thickness and completeness of the black stripe and the size of the black edge of the mesepisternum (mesepisternum colouration, Figure 3); these were treated as one character as they appeared to be intimately correlated. The second character deals with the colour and thickness of the stripe present on the ventral side of the thorax and the area around (ventral colouration, Figure 4). The third character concerns the colouration of the abdominal tergites, being entirely black, with some white laterally or with a white stripe dorsal on the tergite (tergite colouration, Figure 5).

Table 2. Morphological characters scored for *Pachyprotasis rapae.*

Character		**State 1**	**State 2**	**State 3**
1	Mesepisternum colouration	Complete thick stripe Thick black edges	Incomplete thick or medium sized stripe Black edges	Incomplete medium sized or thin stripe Thin black edges
2	Ventral colouration	Very thick black stripe Area mostly black	Thick black stripe Area mostly white	Thin black stripe Area around white
3	Tergite colouration	Black	White laterally	With white stripe

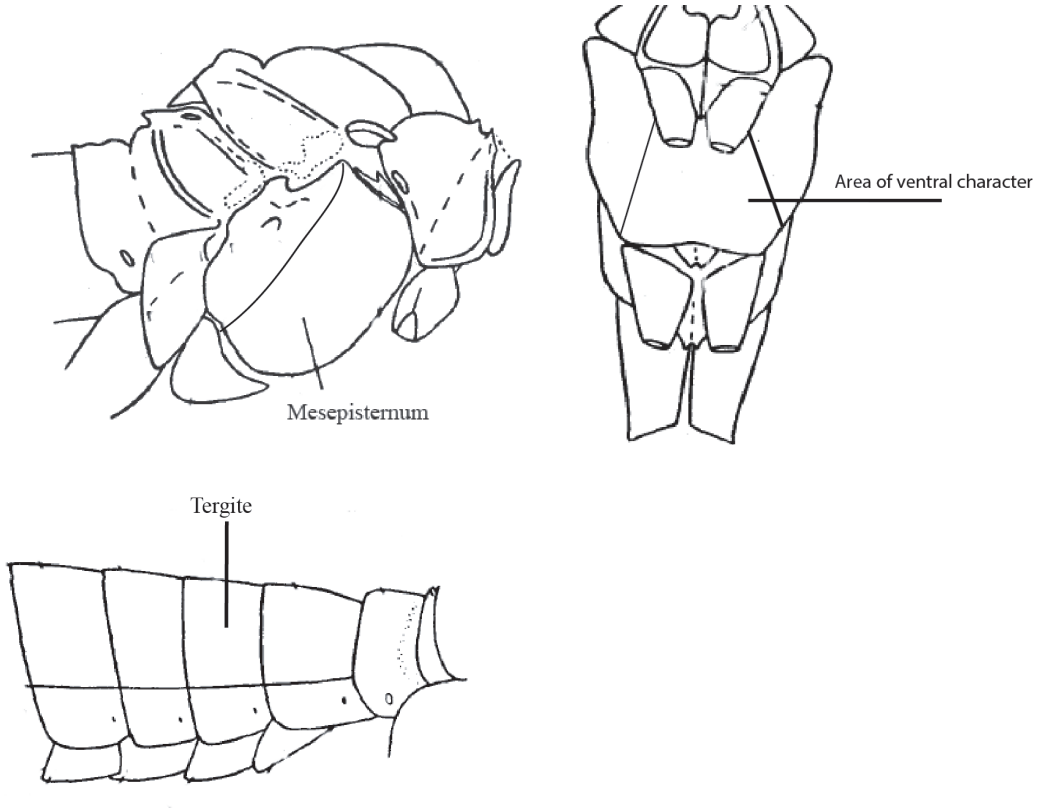

Figure 2. External morphology of sawfly modified from Steyskal (1988). Top left: Thorax, lateral view. Top right: Thorax, ventral view. Bottom: Abdominal segments 1-5, lateral view. Position of characters used in the thesis is marked on figures.

Figure 3. Mesepisternum of *Pachyprotasis rapae* (lateral view), showing patterns of colouration . From left to right: Character state 1, 2, and 3.

Figure 4. Ventral side of thorax for Pachyprotasis *rapae*, showing patterns of colouration. From left to right: Character state 1, 2, and 3.

Figure 5. Tergite of *Pachyprotasis rapae* (dorsal view), showing patterns of colouration . From left to right: Character state 1, 2, and 3.

2.5.2 *Athalia circularis*

There is known variation in the colouration of the mesepisternum in *A. circularis*. There is always a black stripe across the mesepisternum, but the remaining parts of the mesepisternum can range from almost all black to all yellow within a population (Benson 1962, Mol 2009). For *A. circularis* two colour characters were chosen (Figure 2), which were scored with four and two character states, respectively (Table 3). The first character was the amount of black compared to yellow above the black stripe on the mesepisternum (mesepisternum colouration, Figure 6). The second character concerns the amount of black compared to yellow below the stripe and at the ventral side of the thorax (ventral colouration, Figure 7).

Table 3. Explanation of the morphological characters scored for *Athalia circularis.*

Character		**State 1**	**State 2**	**State 3**	**State 4**
1	Mesepisternum colouration	All black	Mostly black Yellow patches	Mostly yellow Black edges and patches	All yellow
2	Ventral colouration	Black stripe extended ventral Edge with a black stripe	Almost all yellow No black except on mesepisternum		

Figure 6. Mesepisternum of *Athalia circularis* (lateral view), showing patterns of colouration. From left to right: Character state 1, 2, 3 and 4. Black colour represents black, and white represents yellow (only in *Athalia* figures).

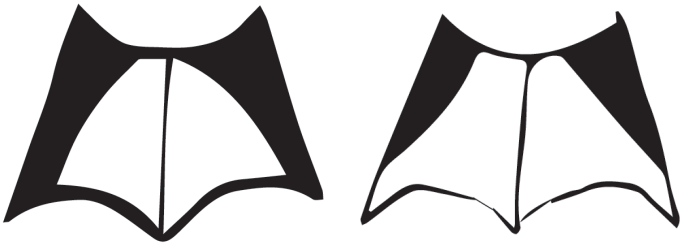

Figure 7. Ventral side of thorax for *Athalia circularis*, showing patterns of colouration. From left to right: Character state 1 and 2. Black colour represents black and white represents yellow (only in *Athalia* figures).

2.5.3 Morphology of genitalia

For each species, five females of each haplogroup were selected for the genitalia study. Each saw was studied without taking into consideration which haplogroup it belonged to. The genitalia of the specimens were removed using thin needles and tweezers. The genitalia were heated in a microwave oven with lactic acid to remove soft tissue. The time needed was depended on the degree of sclerotization. The genitalia were then transferred to 80 % ethanol followed by transfer to a microscope slide with a small amount of glycerol, covered with a cover glass and studied under microscope. Photographs were then taken and then studied in Adobe Photoshop CS6. The comparison of the genitalia was done entirely by visual assessment and no morphometric statistics were used. The focus was on the denticles of the saw and the structures around them.

2.5.4 Statistical analyses of body morphology

Analysis of variance (ANOVA) was done in IBM SPSS Statistics 20.0.0.1 (IBM Corp., Armonk, NY, USA) to test if differences in body morphology exist between local populations defined based on the EIS-system. Independent-samples t-tests were done on the variance of body morphology between the two haplogroups, and between the two sexes in each species. *Pachyprotasis* were divided into five local populations (Table 4). *Athalia* were divided into six local populations (Table 4). List of specimens in the different populations can be found in Appendix 1 (Table 2-A).

Table 4. Local population of *Pachyprotasis rapae* and *Athalia circularis* used in the ANOVA. Definition of local populations was based on the European invertebrate survey (EIS)-system.

Population	n	EIS square(s)
Pachyprotasis rapae		
1	18	36
2a	13	20,29
2b	7	19,27,28
3	13	45,53,54
4	9	37,38
Athalia circularis		
1	18	11
2	14	20
3	14	28
4	14	29
5	12	27,34
6	5	36

3 Results

3.1 *Pachyprotasis rapae*

3.1.1 Sequence analyses

Details about the genetic partitions are given in Table 5. Sequences were searched for numts. This was done by looking for double peaks, stop codons and frame shift mutation in the sequences. No sign of numts were discovered.

The Neighbour-joining analysis of the COI gene divided the *P. rapae* specimens into two haplogroups, each with high bootstrap support (99 % and 97 %, respectively; Figure 8). The same result was found with the Maximum Likelihood analysis (Appendix 2, Figure 1-A). The genetic distance between the two haplogroups was 4 %. The genetic distances between the out-groups and the haplogroups were somewhat larger, 5.5-6.5 % for haplogroup 1 and 4.8-6.6 % for haplogroup 2 (Table 6).

The Neighbour-joining analysis of the CAD sequences did not identify the two haplogroups found with COI (Figure 9), despite the fact that the analysis included five specimens from COI haplogroup 1 and eight specimens from COI haplogroup 2. The same result was obtained by use of the Maximum Likelihood analysis (Appendix 2, Figure 2-A).

There was no geographical structure of specimens within or between the COI haplogroups, as AMOVA showed no significant overall genetic differentiation (Table 7; F_{ST}=0, p-value=0.51), and no significant pairwise differences (all p-values > 0.14±0.03) between the four local geographical populations of *P. rapae* (based on the EIS-system) were found.

Table 5. Polymorphic sites of COI and CAD for *Pachyprotasis rapae*.

Locus	Number of specimens	Number of nucleotide sites	Singleton variable sites	Parsimony informative sites
COI	44	661	44	49
CAD	15	1052	42	27

Table 6. Genetic distances between the two COI haplogroups of *Pachyprotasis rapae* and the out-group taxa (*P. variegata* and *P. antennata*), based on the Tamura 3-parameter model. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates).

	Haplogroup 1	**Haplogroup 2**	***P. variegata***	***P. antennata***
Haplogroup 1	-	0.008	0.009	0.009
Haplogroup 2	0.040	-	0.009	0.010
P. variegata	0.055	0.048	-	0.008
P. antennata	0.065	0.066	0.057	-

Table 7. AMOVA of COI variation in Norwegian *Pachyprotasis rapae*. Four local populations were defined based on the European invertebrate survey (EIS) system (see Table 1 for details).

Source of variation	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among populations	3	20.948	-0.15532 Va	-1.94
Within populations	28	228.583	8.16369 Vb	101.94
Total	31	249.531	7.99517	

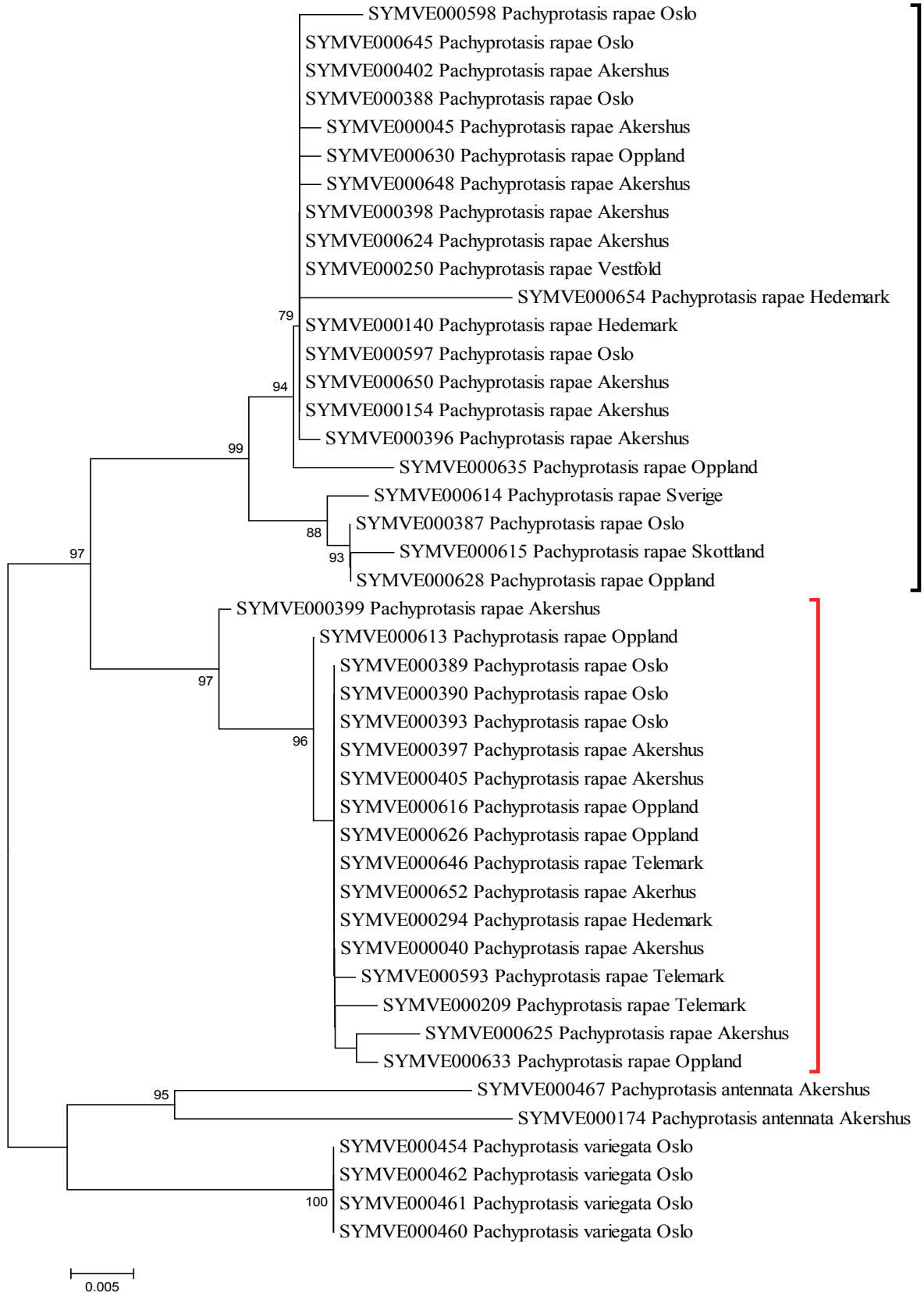

Figure 8. Neighbour-joining analysis performed on 44 COI sequences of *Pachyprotasis* with 1000 bootstrap replicates (only support values above 75 % shown). *P. rapae* haplogroup 1 = black; haplogroup 2 = red.

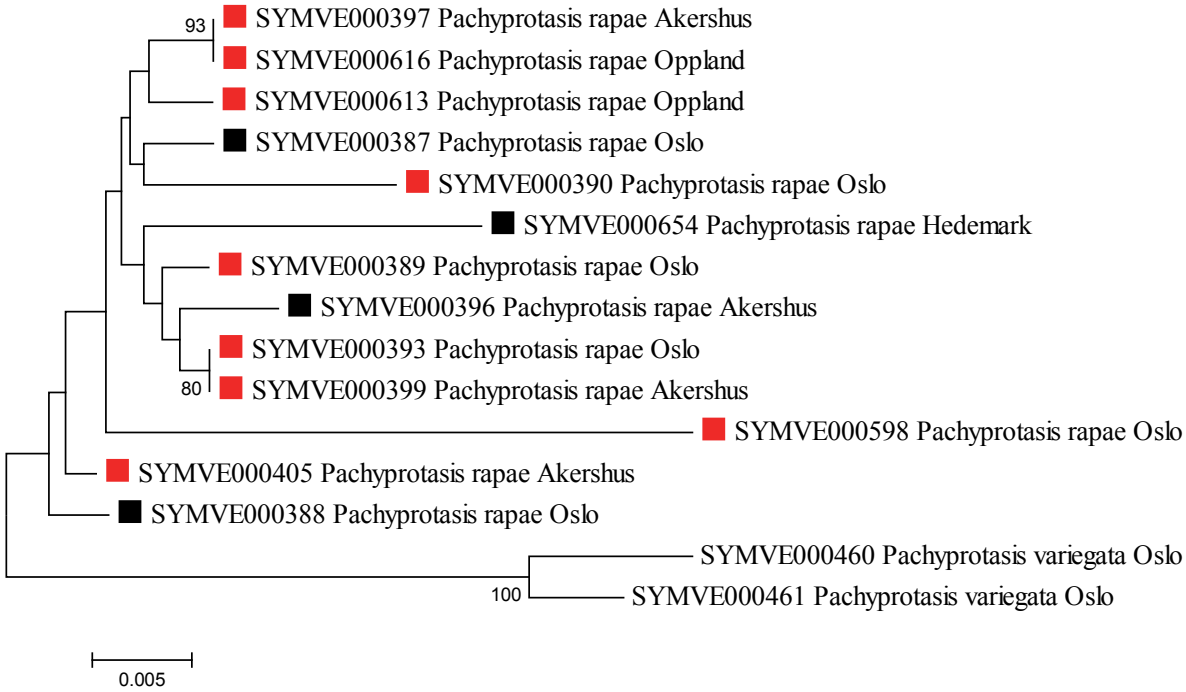

Figure 9. Neighbour-joining analysis performed on 15 CAD sequences of *Pachyprotasis* with 500 bootstrap replicates (only support values above 75 % shown). *P. rapae* specimens *with* COI haplogroup 1 = black squares. *P. rapae* specimens with COI haplogroup 2 = red squares.

3.1.2 Body morphology

The independent-samples t-test did not show any significant colour differences between the two haplogroups (Appendix 2, Table 3-A), nor did the ANOVA show any significant colour differences between the local populations (Appendix 2, Table 4-A). An independent samples t-test on sexes dependent colouration differences showed significant differences in all three characters (Table 8).

Table 8. Independent-samples t-test on body morphology differences between the sexes of *Pachyprotasis rapae*.

	Mesepisternum colouration	Ventral colouration	Tergite colouration
t	2.690	2.662	3.359
df	67	67	67
Sig. (2-tailed)	0.009	0.010	0.001
Mean difference	0.510	0.351	0.663
Std. error difference	0.189	.132	0.198
95 % CI lower limit	0.131	0.088	0.269
95 % CI upper limit	0.888	0.614	1.058

3.1.3 Morphology of genitalia

Detailed description of the saw of each specimen can be found in Appendix 3. In Figure 10 all described structures can be seen. The denticles were numbered from the anterior to the posterior side.

The out-group *P. variegata* had 19 denticles (Figure 11). Denticles 1-3 had reduced or no jagged edges, broad basis and relatively broad apex compared to *P. rapae* (Figure 10). Denticles in the middle section of the saw had clear jagged edges on the posterior side, a broad basis and a broader apex than what was found in *P. rapae.* The last six denticles were smaller and wave-shaped.

The *P. rapae* specimens belonging to haplogroup 1 had saws with 16-18 denticles, with little variation between the individuals. The first few denticles (1-3) had reduced or no jagged edges on the posterior side and a broader apex. The denticles in the middle section of the saw (4-13/14) had jagged edges on the posterior side and 1-2 tips with or without a hump on the anterior side, a broad basis and relatively smaller apex compared to *P. variegata*. The apex-shape of the denticles varied from narrow to round within individuals and between specimens but with no specific pattern. The posterior denticles (14-17) were wave-shaped and had jagged edges on the posterior side and an indent on the anterior side.

The *P. rapae* specimens belonging to haplogroup 2 had saws with 16-18 denticles and a general morphology similar to that of haplogroup 1. The first few denticles (1-3) had reduced or no jagged edges on the posterior side and a broader apex. The denticles in the middle

section of the saw (4-13/14) had jagged edges on the posterior side and 1 or 2 tips combined with or without a hump on the anterior side, a broad basis and relatively smaller apex. The apex-shape varied from narrow or a round apex but with no specific pattern. The posterior denticles (14-17) were wave-shaped and had jagged edges on the posterior side and an indent on the anterior side.

A clear difference in morphology of the female genitalia was observed between *P. rapae* and *P. variegata*. These differences are clearly illustrated by Figure 10 and 11. No differences or clear pattern were observed between the two haplogroups of *P. rapae*.

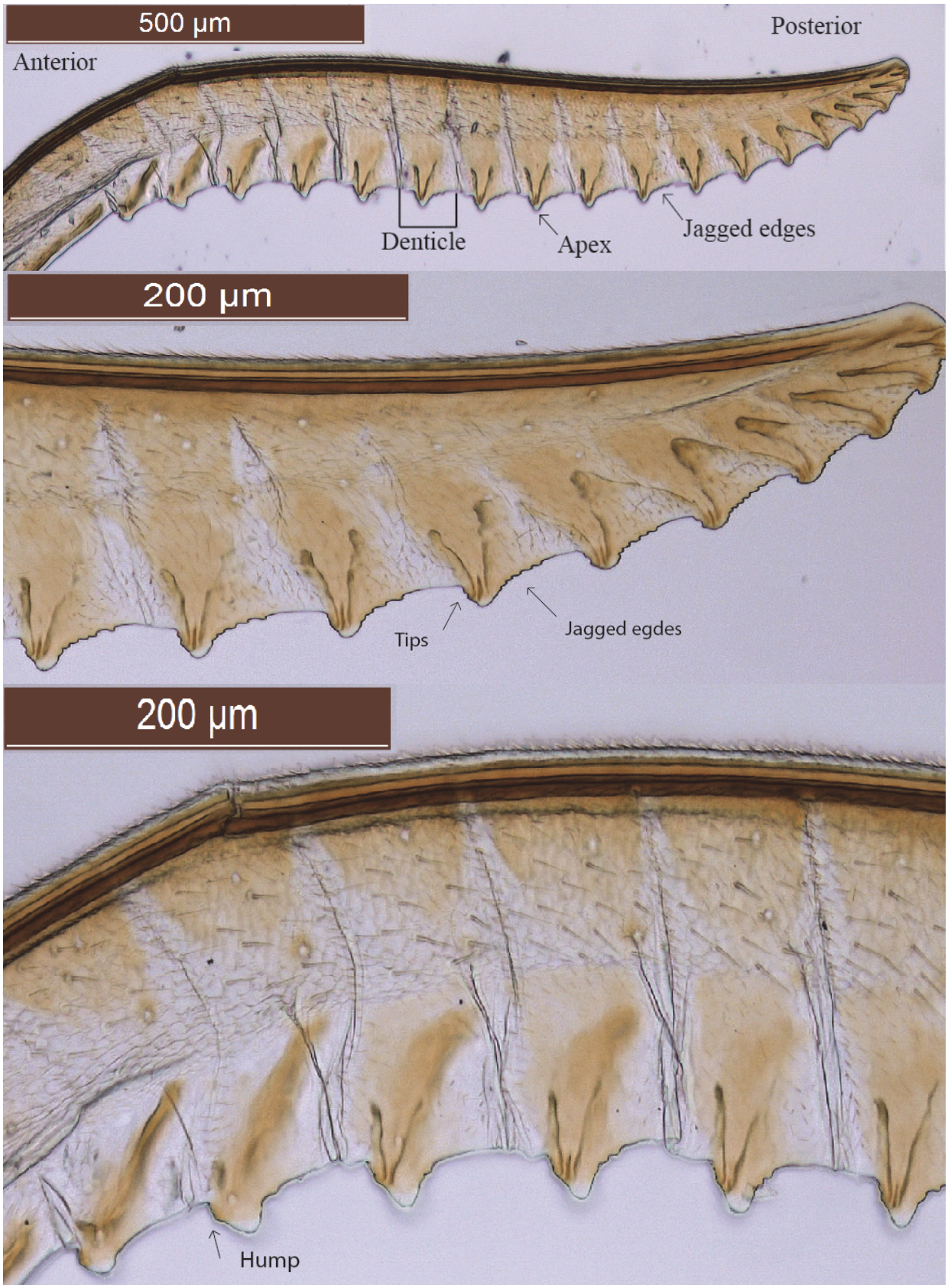

Figure 10. The saw of *Pachyprotasis rapae* (specimen SYMVE000040). Top: Entire saw. Middle: Posterior part of saw. Bottom: Anterior part of the saw. Structures used in description of saw are indicated.

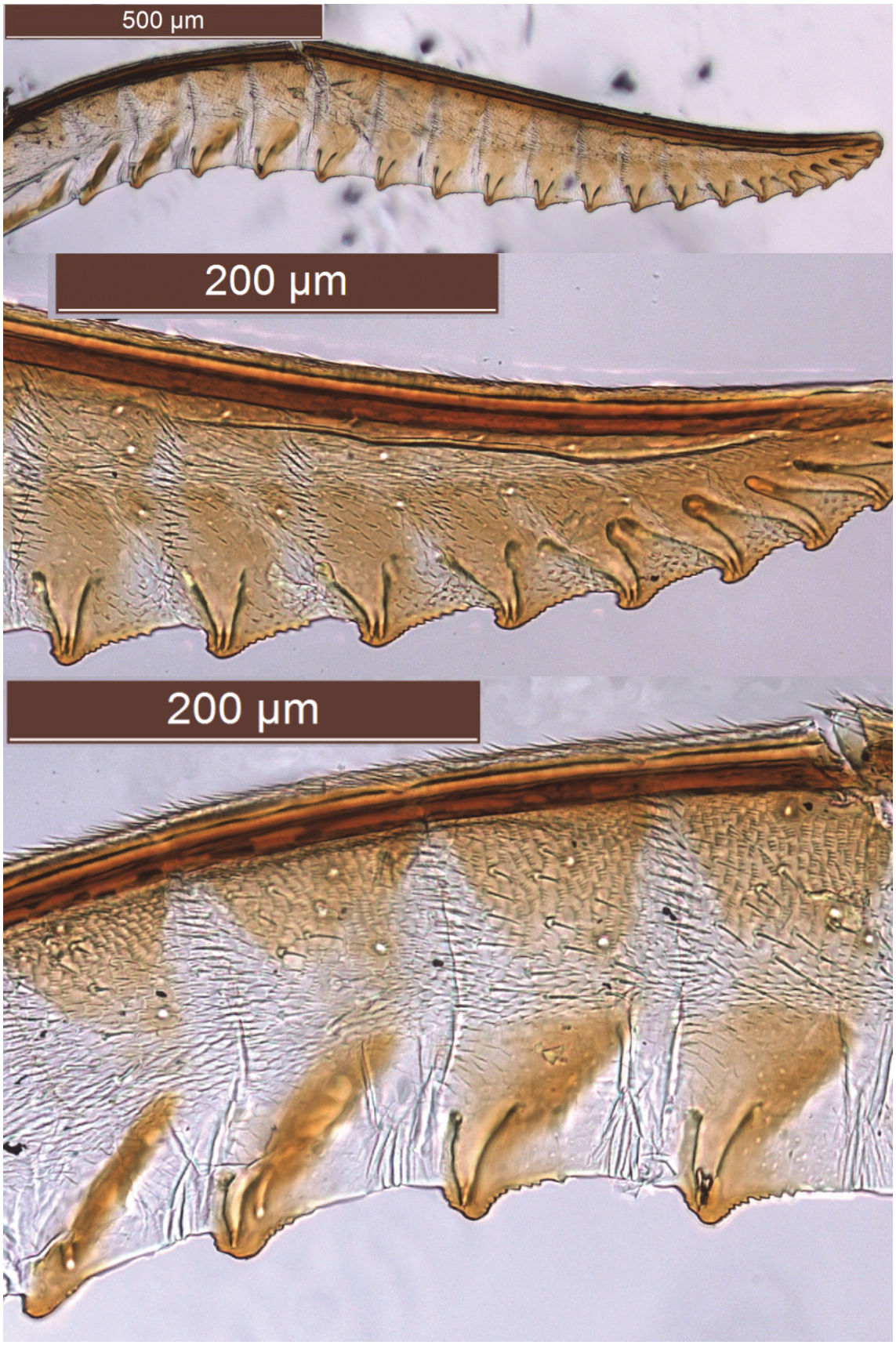

Figure 11. The saw of *Pachyprotasis variegata* (specimen SYMVEV000054). Top: Whole saw. Middle: Posterior end of the saw. Bottom: Anterior part of the saw.

3.2 *Athalia circularis*

3.2.1 Sequence analyses

Details about the genetic partitions are shown in Table 9. Sequences were searched for numts. This was done by looking for double peaks, stop codons and frame shift mutation in the sequences. No sign of numts were discovered.

The Neighbour-joining tree of the COI gene (including only one of the out-group taxa, *A. cordata*) divided *A. circularis* into two haplogroups, with moderate to high bootstrap support (72 % and 99 %, respectively; Figure 12). The same result was obtained by the Maximum Likelihood analysis and the Neighbour-joining analysis including all four out-group taxa (Appendix 2, Figures 3-A and 4-A). The genetic distance between the two haplogroups was 2 %. The genetic distances between the out-group taxa and the haplogroups were considerably larger, 11-13 % for haplogroup 1 and 12-13 % for haplogroup 2 (Table 10).

The Neighbour-joining analysis of the CAD sequences did not identify the two haplogroups found with COI (Figure 13) despite the fact that the analysis included three specimens from COI haplogroup 1 and nine specimens from haplogroup 2. The same result was obtained by the Maximum Likelihood analysis (Appendix 2, Figure 5-A).

AMOVA based on COI sequences of six local populations of *A. circularis* (based on the EIS-system) showed larger variation within populations than among them (Table 11); however the analyses gave a significant result for genetic differences among populations (F_{ST}=0.110, p-value=0.00880). Calculation of pairwise F_{ST} showed that there were significant pairwise differences between several of the local populations with standard critical probability level (p-values < 0.05, Table 12), but with the Bonferroni correction (Rice 1989) there was only one significant result (the pairwise difference between population 4 and population 6).

Table 9. Polymorphic sites of COI and CAD for *Athalia circularis.*

Locus	Number of specimens	Number of nucleotide sites	Singleton variable sites	Parsimony informative sites
COI	91	660	19	143
CAD	15	1074	17	30

Table 10. Genetic distances between the two COI haplogroups of *Athalia circularis* and the out-group taxa (*A. cordata*, *A. liberta*, *A. lugens* and *A. rosea*). Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates).

	Haplogroup 1	**Haplogroup 2**	***A. cordata***	***A. liberta***	***A. lugens***	***A. rosea***
Haplogroup 1	-	0.005	0.015	0.016	0.014	0.016
Haplogroup 2	0.020	-	0.016	0.017	0.015	0.016
A. cordata	0.117	0.121	-	0.017	0.017	0.017
A. liberta	0.129	0.131	0.149	-	0.015	0.013
A. lugens	0.117	0.121	0.140	0.098	-	0.014
A. rosea	0.134	0.128	0.154	0.091	0.106	-

Table 11. AMOVA of COI variation in Norwegian *Athalia circularis*. Definition of the six local populations were based on the European invertebrate survey (EIS)-system (see Table 1 for details).

Source of variation	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among populations	5	45.755	0.46487 Va	11.01
Within populations	65	244.133	3.75589 Vb	88.99
Total	70	289.887	4.22075	

Table 12. Pairwise F_{ST} values and p-values of six local populations of *Athalia circularis* defined based on the European invertebrate survey (EIS)-system (see Table 1 for further explanation). Top half: + = pairwise F_{ST} values between populations. Bottom half: pairwise F_{ST} p-values.

	1	2	3	4	5	6
1		0.113	0.081	0.185	-0.055	0.272
2	0.054±0.024		0.015	-0.018	0.112	0.360
3	0.081±0.025	0.297±0.049		0.039	0.048	0.091
4	0.009±0.009	0.396±0.047	0.225±0.038		0.180	0.315
5	0.837±0.040	0.126±0.027	0.171±0.031	0.009±0.009		0.272
6	0.036± 0.020	0.018±0.012	0.207±0.027	0.000±0.000	0.009±0.009	

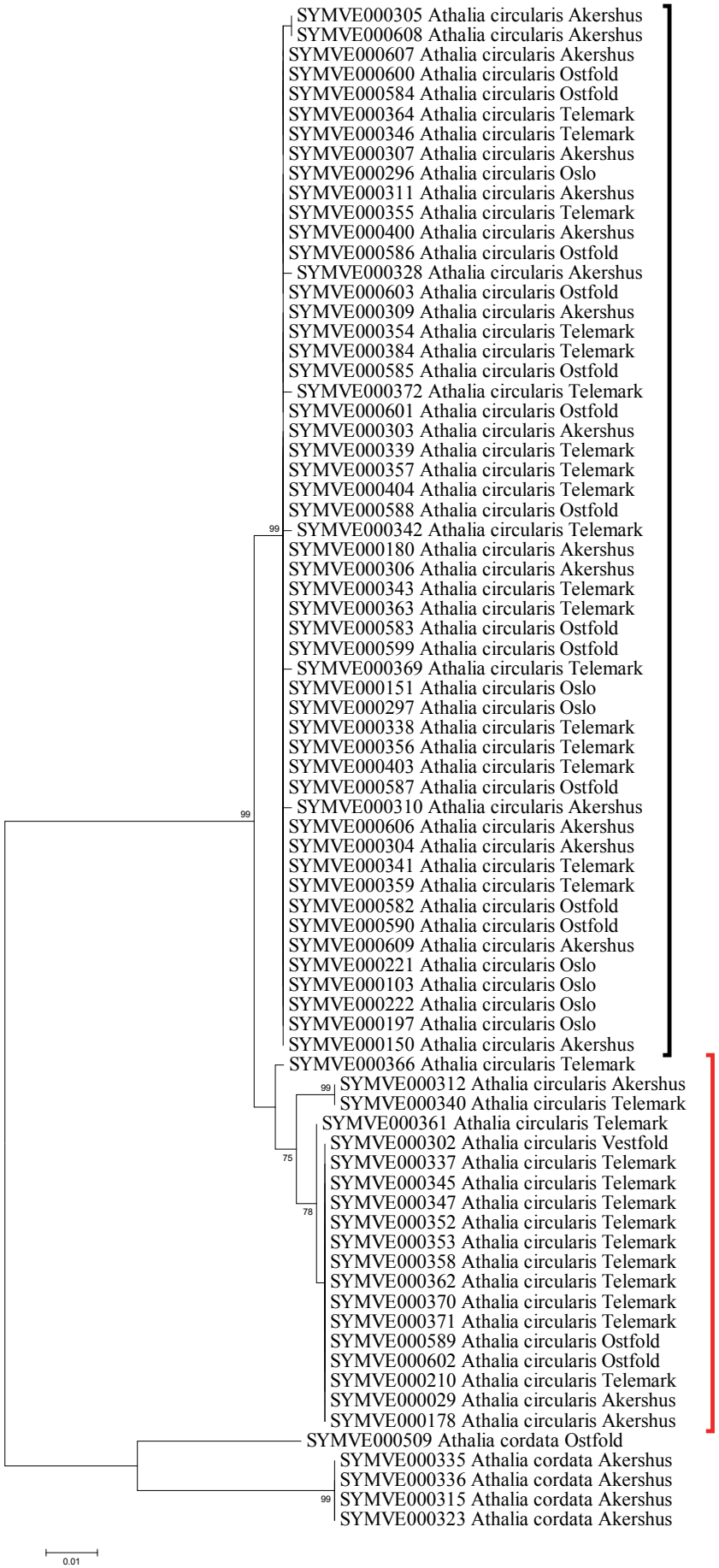

Figure 12. Neighbour-joining analysis performed on 77 COI sequences of *Athalia* with 1000 bootstrap replicates (only support values above 75 % shown). *A. circularis* haplogroup 1 = black; haplogroup 2 = red. Only one out-group taxon is included in the analysis.

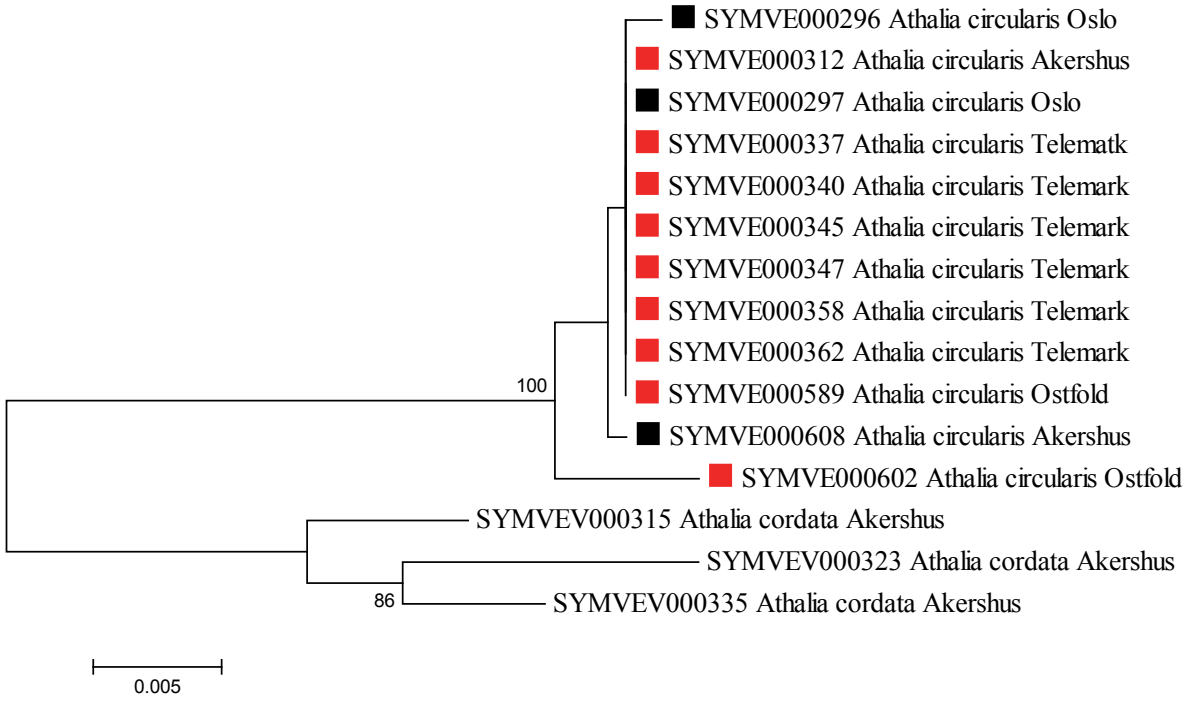

Figure 13. Neighbour-joining analysis performed on 15 CAD sequences of *Athalia* with 500 bootstrap replicates (only support values above 75 % shown). *A. circularis* specimens with COI haplogroup 1 = black squares. *A. circularis* specimens with COI haplogroup 2 = red squares.

3.2.2 Body morphology

An independent-samples t-test did not show any significant difference in the colouration between the two COI haplogroups (Appendix 2, Table 5-A). The ANOVA gave a significant result for body colouration differences between the local populations (Table 13). The independent-samples t-test for sex dependent body colouration differences gave significant result for mesepisternum colouration; however there was no significant result for the ventral colouration (Table 14).

Table 13. ANOVA of body morphology in local populations of *Athalia circularis* (populations defined by the European invertebrate survey (EIS)-system; see Table 4 for details).

	Sum of Squares	df	Mean Square	F	Sig.
Mesepisternum colouration					
Between populations	34.208	5	6.842	8.054	0.000
Within populations	60.311	71	0.849		
Total	94.519	76			
Ventral colouration					
Between populations	5.596	5	1.119	7.040	0.000
Within populations	11.287	71	0.159		
Total	16.883	76			

Table 14. Independent-samples t-test on body morphology differences between the sexes of *A. circularis*.

	Mesepisternum colouration	Ventral colouration
t	0.639	-2.575
df	70.710	72.662
Sig. (2-tailed)	0.525	0.012
Mean difference	0.160	-0.259
Std. error difference	0.250	0.101
95 % CI lower limit	-0.339	-0.459
95 % CI upper limit	0.658	-0.059

2.2.3 Morphology of genitalia

Detailed description of the saw of each specimen can be found in Appendix 3. The described structures can be seen in Figure 10. The denticles were number from the anterior to the posterior side.

The out-group taxon *A. cordata* (Figure 14) had a saw with a total of 15 denticles. The denticles had a narrow basis and a very narrow apex, and were long compared to the denticles of *A. circularis* (Figure 15). The pattern above every denticle varied from a V-shape to a single line-shape figure. The anterior positioned denticles (1-2/3) had broad apex with a slight slope on the anterior side and a straight posterior side. The denticles of the middle section of the saw (2/3-10) had narrow apex with straighter anterior and posterior sides; the posterior

side with jagged edges. The last denticles (11-15) had slight slope on the anterior side and a straighter posterior side, with reduced jagged edges.

The *A. circularis* specimens belonging to haplogroup 1 had saws with 16-17 denticles, and little variation between individual denticles. There was some variation in the pattern above the denticles, but with no shared general pattern among specimens. The anterior positioned denticles (1-3) were smaller of size, had a slight slope on the anterior side and straighter posterior side; the posterior side with reduced or no jagged edges. The denticles in the middle section of the saw (4-12-/13) had a slight slope and jagged edges on both sides. The last few denticles (13-17) had a slight slope on the anterior side and straighter posterior side, the posterior side with jagged edges.

The *A. circularis* specimens belonging to haplogroup 2 had saws with 16-17 denticles and little variation between the denticles. There was some variation in the pattern above the denticles, but with no shared general pattern among specimens. The anterior positioned denticles (1-3) were smaller of size and had a slight slope on the anterior side, with straighter posterior side, reduced or no jagged edges. The denticles in the middle section of the saw (4-12-/13) had a slight slope and jagged edges on both sides. The last few denticles (13-17) had a slight slope on the anterior side, but a straighter posterior side, the posterior side with jagged edges.

There was a clear difference between the female genitalia of *A. cordata* and *A. circularis*. Within *A. circularis* there was no clear difference observed between specimens of the two haplogroups. The variation found in the pattern above the denticles did not correlate with the haplogroups.

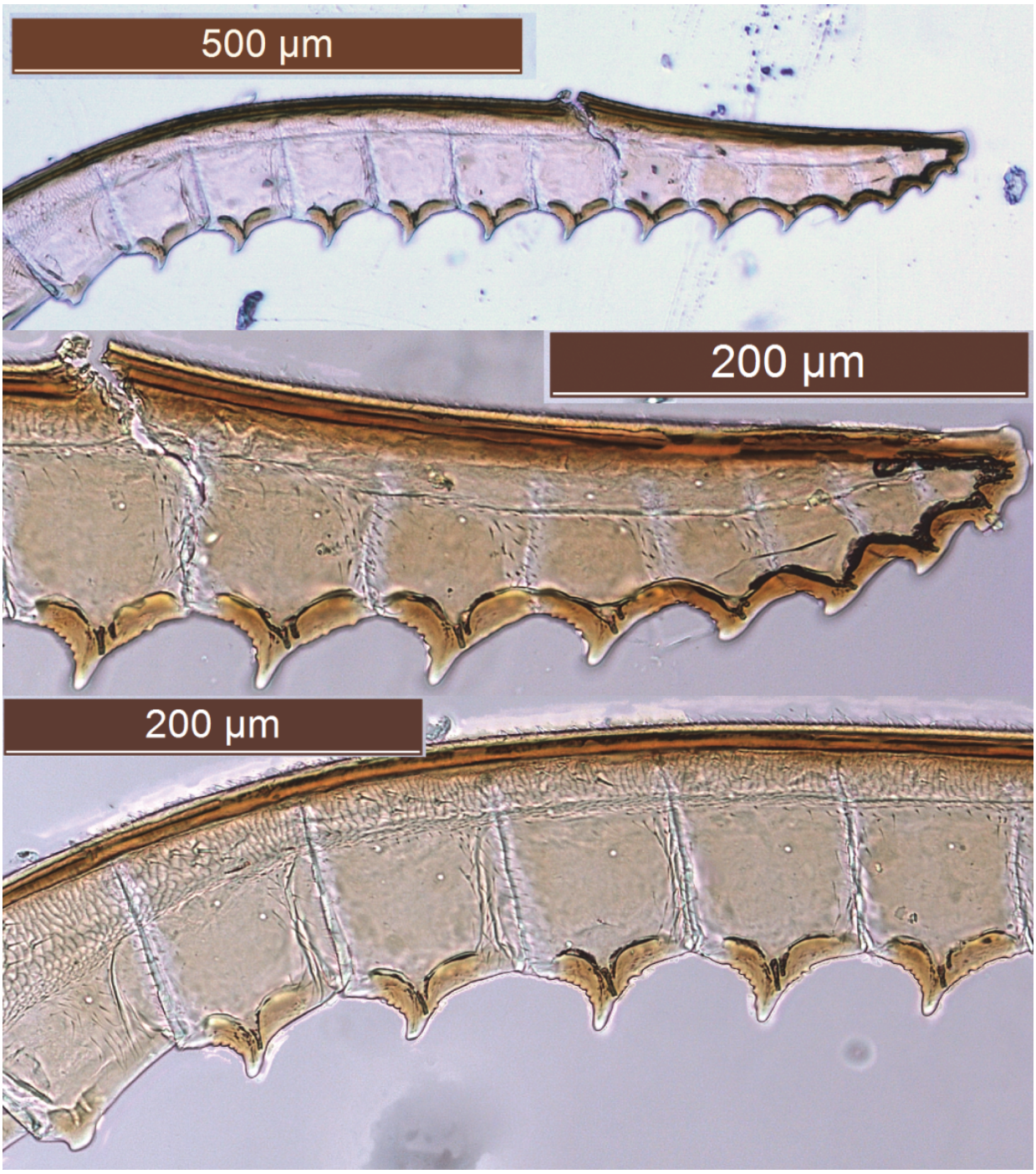

Figure 14. The saw of *Athalia cordata* (specimen SYMVEV000335). Top: Whole saw. Middle: Posterior end of the saw. Bottom: Anterior part of the saw.

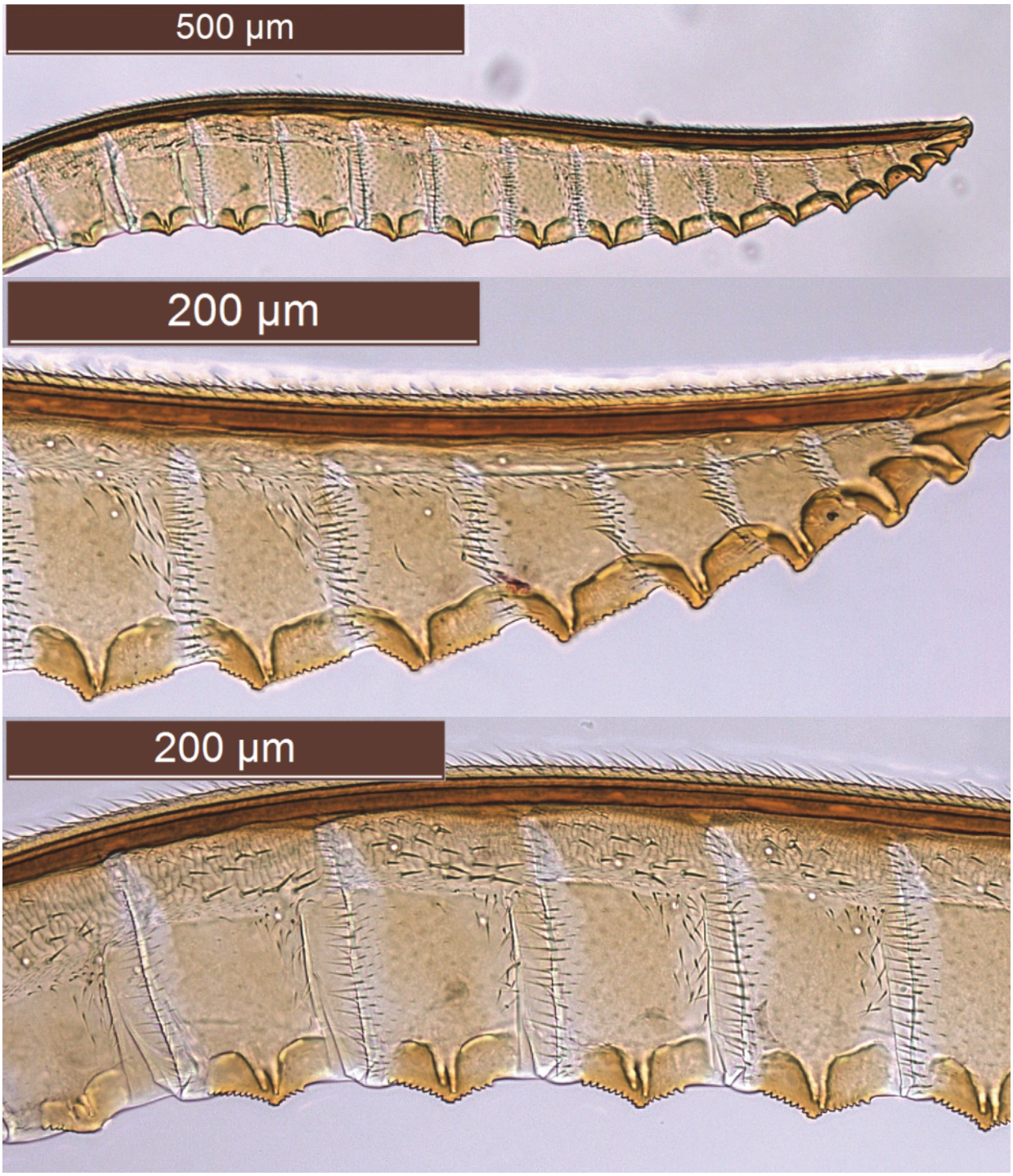

Figure 15. The saw of *Athalia circularis* (specimen SYMVEV000590). Top: Whole saw. Middle: Posterior end of the saw. Bottom: Anterior part of the saw.

4 Discussion

4.1 *Pachyprotasis*

The analyses of mtDNA (COI) including a larger sample of Norwegian *P. rapae* specimens reveals the existence of two haplogroups (separated by a genetic distance of 4 %) in line with previous evidence indicated in a DNA barcoding project (Lønnve, Lifjeld and Johnsen, unpublished). The two haplogroups are, however, not corroborated by nDNA sequences (the CAD region) for a selected number of specimens. The existence of high intraspecific mtDNA variation in animals has in some cases been explained by the existence of cryptic species (Hebert et al. 2004, Barrett and Hebert 2005, Hajibabaei et al. 2006). If the two haplogroups in *P. rapae* represent true cryptic species, we would expect to find corresponding intraspecific variation in nDNA. This is not the case and most likely cryptic species do not account for the intraspecific mtDNA variation found in *P. rapae*.

There are no obvious patterns in the geographical distribution of the *P. rapae* specimens within or between the COI haplogroups. The AMOVA based on local populations (defined based on the EIS-system) shows that variation within populations is much larger (101.94) than among populations (-1.94). The negative value for the variation among populations could be due to a statistical artifact associated with lack of variation between the groups. Negative values should be assessed as zero and follow with a non-significant p-value (Long 1986, Excoffier et al. 1992).

Body colouration and morphology of the female genitalia were analysed to see if intraspecific variation in any of these characters corresponds with the two COI haplogroups. Intraspecific variation in body colouration exists within *P. rapae*, but according to the independent-samples t-test there are no significant colouration differences between the two haplogroups. Variation in the female genitalia has previously been used to distinguish between closely related Symphyta species (Heidemaa et al. 2004, Prous et al. 2011). Some variation in the number and shape of denticles on the saw is found among the investigated specimens, but no clear differences between the two haplogroups are revealed.

The body colouration differences found within *P. rapae* do not correspond significantly to the local populations (defined based on the EIS-system). However, variation within all three

characters (mesepisternum colouration, ventral colouration and tergite colouration) corresponds significantly with the specimen sex. The males seem to be overall darker than the females. The result should, however, be interpreted carefully as sampling bias is most likely introduced because of the small sampling sizes of several of the populations. None of the datasets are normally distributed and all of the local populations would most likely constitute a single East Norwegian population in a study at a larger geographical scale. Nevertheless, the results can be used as a starting point for generating new hypotheses for future studies about differences in body colouration within *P. rapae*.

The two COI haplogroups found within *P. rapae* do not correspond with variation in nDNA, geography, body colouration or morphology of the female genitalia. There is, thus, no support for the hypothesis that the intraspecific mtDNA variation represents different (cryptic) species. What then could be the reason behind the intraspecific mtDNA variation? Other reasons have been proposed to explain intraspecific mtDNA variation. One alternative explanation is numts, the transfer of mtDNA to the nDNA. This has been shown in other studies to create an artificially high estimate of unique species (Gellissen et al. 1983, Song et al. 2008, Buhay 2009). Universal primers used to amplify the same sequence across a wide range of taxa (like the primers used in this study, Folmer et al. 1994) do not necessarily discriminate between numts and mtDNA. However no stop codons, double peaks or frame shift mutations were discovered in the *P. rapae* sequences, which most likely rules out numts as the explanation behind the observed mtDNA divergence. Second, introgression via hybridization with closely related species may also introduce intraspecific variation within mtDNA, but with no or almost no corresponding introgression of the nDNA (Sota 2002, Linnen and Farrell 2008, Zakharov et al. 2009). None of the haplogroups share haplotypes with the two out-group taxa, which are congeneric species of *P. rapae* and as such likely candidates for mtDNA exchange via introgression, but more thorough investigation is needed to rule out that introgression from other *Pachyprotasis* species might have taken place. Further, ancient introgression from species that are now extinct, could also result in the same patterns of incongruence between mtDNA and nDNA (Ballard and Whitlock 2004). Third, the cytoplasmic endosymbiont *Wolbachia* may introduce intraspecific variation within mtDNA leading to incongruence between mtDNA and nDNA (Kvie et al. 2012). Smith et al. (2012) showed that almost 1 % of Hymenoptera species available in The Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007) have signs of *Wolbachia* infection. The attempt to amplify WSP from *P. rapae* as part of this thesis gave no product; however, no

positive controls were available, hence presence or not of *Wolbachia* in *P. rapae* is unresolved. Fourth, different rates of lineage sorting in isolated populations may also lead to intraspecific variation and incongruence between mtDNA and nDNA (Maddison 1997, Freeland 2005). A constant molecular clock (Kimura 1968) has been suggested for mtDNA, with 0.02 substitutions per base pair (or 2 % differences) per million years (Brown et al. 1979, Avise 1986, Desalle et al. 1987), but the idea of a constant molecular clock is controversial (Lynch and Jarrell 1993, Galtier et al. 2009, Nabholz et al. 2009). A total of 4 % difference between the two haplogroups would be consistent with two million years of divergence according to this theory. Several areas in the northern hemisphere were affected by the Pleistocene ice ages (2.58-0.01 million years ago) (Farmer and Cook 2013) and geographical separated refugia were formed (Hewitt 1996, Chang et al. 2007) . Several studies have shown that refugia worked as isolation barriers between populations, which became genetically diverged (Hewitt 1996, Knowles 2000, Ribera and Vogler 2004, Chang et al. 2007). If diverged populations from different refugia had not yet evolved reproductive barriers, secondary contact between them would result in the presence of different mtDNA haplogroups in the population. Isolation followed by secondary contact could also affect patterns of nDNA. Differences that accumulate during isolation will upon secondary contacted result in higher variation and less structured nDNA (Hogner et al. 2012). This corresponds well with the patterns of nDNA in *P. rapae*. Although isolation followed by secondary contact seems the most likely explanation for the observed intraspecific variation, it could be that the two haplotypes have been under local selection pressure, which would accelerate the divergence between them (Irwin 2012).

4.2 *Athalia*

As with *P. rapae,* genetic analysis of mtDNA for *A. circularis* divides the specimens into two different haplogroups (separated by a genetic distance of 2 %), in accordance with results from the preliminary DNA barcoding project (Lønnve, Lifjeld and Johnsen, unpublished). The two haplogroups are, however, not corroborated by nDNA sequences (the CAD region) of selected specimens. These results clearly show that there is no consistency between the intraspecific variation in mtDNA and nDNA within *A. circularis*, and as for *P. rapae*, cryptic species is most likely not the reason behind the intraspecific mtDNA divergence.

Even though most of the COI variation is found within local populations (defined based on the EIS system), the AMOVA shows that more variation is found among populations (11.01 %, with low but significant F_{ST} value) than is the case for *P. rapae*. After Bonferroni correction (Rice 1989) only one significant pairwise difference remains, between population 4 and population 6. There is short geographical distance between these two populations and the EIS squares used to define them are diagonally relative to each other. So there is no clear biological explanation as to why these two populations differ from each other. The same caution as with *P. rapae* should be used in interpretation of the results. None of the datasets are normally distributed and the locally defined populations would probably constitute one East Norwegian population in a larger scale study.

Body colouration and morphology of the female genitalia were analysed to see if intraspecific variation in any of these characters corresponds with the two COI haplogroups. Colour differences within *A. circularis* have previously been noted (Benson 1962, Mol 2009), however according to the independent-samples t-test analysis there are no colouration differences between the two COI haplogroups. There is very little variation in the female genitalia of the investigated *A. circularis* specimens; only slight variation in number of denticles on the saw, but these differences is not related to the two COI haplogroups.

The two sexes of *A. circularis* differ significantly in colouration of the ventral side of the thorax. The females seem to be darker dorsally than the males. However, there is no sex related differences in mesepisternum colouration. According to the ANOVA, the body colouration of *A. circularis* differs significantly among the local populations with regard to both characters (mesepisternum colouration and ventral colouration). There seems to be some local populations that are darker than the others. These results should, however, be carefully interpreted because of sampling bias with regard to local population sizes. Sampling bias may lead to false positive results, because the number of individual varies and the data are not normally distributed. As mentioned above, the locally defined *A. circularis* populations will most likely constitute one East Norwegian population in a larger scale study.

The two COI haplogroups found within *A. circularis* do not correspond with variation in nDNA, geography, body colouration or morphology of the female genitalia. As for *P. rapae* there is, thus, no support to the hypothesis that the intraspecific mtDNA variation represents different (cryptic) species. Neither does the intraspecific mtDNA variation seem to result from numts, the transfer of mtDNA to the nDNA (Gellissen et al. 1983, Song et al. 2008). The

COI sequences of both haplogroups have no signs of stop codons, double peaks or frame shift mutations. Signs of introgression via hybridization (as shared haplotypes) are not found between *A. circularis* and the four out-group taxa. This does, however, not rule out the possibility of introgression from other (not investigated) *Athalia* species or ancient introgression involving species which are now extinct. To see if some of the intraspecific mtDNA variation in *A. circularis* could be explained by *Wolbachia* infection, WPS was attempted amplified but gave no product. However, as for *P. rapae* no positive control were available, and the presence or not of *Wolbachia* in *A. circularis* is unresolved. Finally, the COI haplogroups in *A. circularis* have a genetic distance at 2 %. With a constant molecular clock (Kimura 1968) and a 0.02 substitutions per base pair (or 2 % differences) per million years (Brown et al. 1979, Avise 1986, Desalle et al. 1987), this difference would be consistent with one million year divergence between the two haplogroups. This is well within the time of the Pleistocene ice ages (2.58-0.01 million years ago) (Hewitt 1996, Knowles 2000, Farmer and Cook 2013) and the two COI haplogroups could have resided in different refugia. Different rates of lineage sorting in isolated populations, followed by secondary contact may explain the incongruence between mtDNA and nDNA. For large populations it is possible to harbour more than one haplotype of COI (Webb et al. 2011, Hogner et al. 2012).

4.3 DNA Barcoding

By the use of a standardized genetic sequence that shares unique features among members of a species, one of the main aims of DNA barcoding is to identify new species through screening of unknown biodiversity (Hebert et al. 2003a). Used properly, DNA barcoding is an excellent tool to get an overall picture of species groups, and to identify interesting diversity worthy of further study. Indeed, the standard mtDNA marker for the metazoan kingdom, COI, has been used to separate closely related species and to resolve cryptic species complexes (e.g. Hebert et al. 2004, Heidemaa 2004, Hajibabaei et al. 2006, Rasmussen et al. 2009, Steinke et al. 2009). However, relying on COI by itself may lead to erroneous conclusions. Several studies have shown that COI can have highly divergent haplotypes, even within sympatric populations (Chang et al. 2007, Avtzis et al. 2008, Webb et al. 2011, Hogner et al. 2012, Kvie et al. 2012). The results from this thesis thus corroborates a range of previous studies, as both *P. rapae* and *A. circularis* contain large intraspecific variation, with two divergent haplogroups of COI observed within populations of each species.

A phylogenetic analysis based solely on mtDNA can been criticized for good reasons, as it will result in a gene tree that tells the history of the mtDNA only and not the full history of the species (Doyle 1992, Nichols 2001). Accordingly, many authors have advocated the use of more molecular markers when using DNA barcoding for the purpose of taxonomy (Damm et al. 2010, Prous et al. 2011, Dupuis et al. 2012, Van Nieukerken et al. 2012). The use of the CAD gene as a second marker for selected specimens of the two COI haplotypes in *P. rapae* and *A. circularis*, respectively, did not corroborate the patterns observed with mtDNA and thus illustrates the need of more genetic markers to make reliable conclusions. Without the addition of more molecular markers, DNA barcoding could inflate or deflate species numbers. In addition to the problem of identifying cryptic species, COI may sometimes not be able to separate between good morphological species. The work of Prous et al. (2011) on *Empria* (another Tenthredinidae genus) showed that COI is not a suitable marker for species recognition within the *E. longicornis group* and possibly neither within the *E. immerse* group. They suggested ITS1 (internal transcribed spacer 1) as an alternative species-specific marker, as it had better resolution for some groups. In contrast, within the sawfly barcoding project at NHM Oslo, the vast majority of species formed unique clusters, clearly separated from closely related species (Lønnve, Lifjeld and Johnsen, unpublished). Furthermore, it should be noted that species identification using the DNA barcoding library is feasible for the two species in this thesis, as both species form monophyletic groups that are clearly separated from their closely related out-group taxa.

5 Conclusion and further prospects

Neither of the two species, *P. rapae* and *A. circularis*, show any signs of harbouring cryptic species. The intraspecific mtDNA variation observed for both species is not corroborated by either nDNA, local population structure or morphology. Both species, thus, most likely constitute genetically variable species. More studies including a larger geographical sampling area are needed to investigate if the same or other haplogroups exist in the distribution area of the two species, as both species are distributed from Europe to East Asia (Benson 1958, Steyskal 1988). Analyses of other nDNA markers will be important as the CAD-gene could be evolving too slowly to show any differences. As ITS1 is used successfully in other Tenthredinidae genus (Heidemaa 2004, Cook et al. 2011), this genetic marker is a good candidate to be used in further studies on Tenthredinidae genera.

COI can advantageously be used as a molecular marker in initial screening of taxa but should preferably be followed up with a second marker or markers for trustworthy results and to avoid an artificial increase in species numbers. Although the hypothesis of cryptic species was rejected for *P. rapae* and *A. circularis*, it is still important to investigate other species complexes, in Symphyta and other taxa, by the use of DNA barcoding and through this gain greater knowledge about species diversity, including potential cryptic species.

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Appendix 1

Table 1-A. Collection information of all *Athalia* and *Pachyprotasis* specimens analysed in the thesis.

Journal No	Species	Sex	Collector	Date	Latitude	Longitude	Locality	County	Country
SYMVE000017	*Athalia liberta*	Female	Lønnve, O. J.	1.IX.2007	59.51	10.40	Nesodden	Akershus	Norway
SYMVE000029	*Athalia circularis*	Female	Lønnve, O. J.	9.V.2007	59.51	10.40	Skoklefall	Akershus	Norway
SYMVE000040	*Pachyprotasis rapae*	Female	Lønnve, O. J.	9.VI.2007	59.49	10.41	Røer	Akershus	Norway
SYMVE000045	*Pachyprotasis rapae*	Female	Lønnve, O. J.	9.VI.2009	59.49	10.41	Røer	Akershus	Norway
SYMVE000060	*Athalia rosae*	Female	Lønnve, O. J.	6.VI.2007	59.54	10.44	Hovedøya	Oslo	Norway
SYMVE000103	*Athalia circularis*	Female	Endrestøl, A.; Hansen, L. O.	8.VIII.2007	59.53	10.46	Ekebergskråninga	Oslo	Norway
SYMVE000140	*Pachyprotasis rapae*	Male	Sund, K.	28.VI.2007	60.12	12.27	Vikeråa	Hedemark	Norway
SYMVE000150	*Athalia circularis*	Female	Endrestøl, A.; Hansen, L. O.	28.VI.2007	59.60	10.46	Kirkeby	Oslo	Norway
SYMVE000151	*Athalia circularis*	Female	Endrestøl, A.; Hansen, L. O.	28.VI.2007	59.60	10.46	Kirkeby	Oslo	Norway
SYMVE000154	*Pachyprotasis rapae*	Male	Endrestøl, A.; Hansen, L. O.	28.VI.2007	59.6	10.46	Kirkeby	Oslo	Norway
SYMVE000174	*Pachyprotasis antennata*	Female	Lønnve, O. J.	5.VI.2008	59.51	10.40	Skoklefall	Akershus	Norway
SYMVE000178	*Athalia circularis*	Female	Lønnve, O. J.	26.V.2008	59.51	10.40	Skoklefall	Akershus	Norway
SYMVE000180	*Athalia circularis*	Female	Lønnve, O. J.	8.VI.2008	59.51	10.41	Ursvik	Akershus	Norway
SYMVE000197	*Athalia circularis*	Male	Lønnve, O. J.	20.VI2008	59.59	10.37	Jegersborg	Oslo	Norway
SYMVE000209	*Pachyprotasis rapae*	Female	Lønnve, O. J.	13.VII-1.VIII.2008	59.939	9.034	Spjeldset	Telemark	Norway
SYMVE000210	*Athalia circularis*	Male	Lønnve, O. J.	13.VII.-1.VIII.2008	59.939	9.034	Spjeldset	Telemark	Norway
SYMVE000221	*Athalia circularis*	Female	Endrestøl, A.; Hansen, L. O.	31.VIII.-13.XII.2007	59.996	10.759	Kirkeby,	Oslo	Norway
SYMVE000222	*Athalia circularis*	Female	Endrestøl, A.; Hansen, L. O.	31.VIII.-13.XII.2007	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000250	*Pachyprotasis rapae*	Male	Olberg, S.	29.V-25.VI.2008	59.319	10.523	Slagentangen	Vestfold	Norway
SYMVE000276	*Athalia lugens*	Female	Olberg, S.	1.VIII.-5.IX.2008	59.319	10.523	Slagentangen	Vestfold	Norway
SYMVE000294	*Pachyprotasis rapae*	Female	Lønnve, O. J.	17.VI.2010	60.887	11.001	Høsbjør	Hedemark	Norway
SYMVE000295	*Athalia circularis*	Female	Olberg, S.; Gammelmo, Ø.	10.VII-27.VII.2011	59.920	10.631	Lilleaker	Oslo	Norway
SYMVE000296	*Athalia circularis*	Female	Olberg, S.; Gammelmo, Ø.	10.VII-27.VII.2012	59.920	10.631	Lilleaker	Oslo	Norway

SYMVE000297	*Athalia circularis*	Male	Olberg, S.; Gammelmo, Ø.	10.VII-27.VII.2013	59.920	10.631	Lilleaker	Oslo	Norway
SYMVE000298	*Athalia liberta*	Male	BioFokus (ØG/SO/KMO)	26.VI-27.VII.2011	58.968	9.845	Larvik	Vestfold	Norway
SYMVE000299	*Athalia lugens*	Female	BioFokus (ØG/SO/KMO)	26.VI-27.VII.2011	58.968	9.845	Nevtungstranda	Vestfold	Norway
SYMVE000300	*Athalia lugens*	Male	BioFokus (ØG/SO/KMO)	26.VI-27.VII.2011	58.968	9.845	Nevtungstranda	Vestfold	Norway
SYMVE000301	*Athalia liberta*	Female	BioFokus (ØG/SO/KMO)	26.VI-27.VII.2011	58.968	9.845	Larvik	Vestfold	Norway
SYMVE000302	*Athalia circularis*	Male	BioFokus (ØG/SO/KMO)	26.VI-27.VII.2011	58.968	9.845	Nevtungstranda	Vestfold	Norway
SYMVE000303	*Athalia circularis*	Female	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000304	*Athalia circularis*	Female	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000305	*Athalia circularis*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000306	*Athalia circularis*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000307	*Athalia circularis*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000308	*Athalia circularis*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000309	*Athalia circularis*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000310	*Athalia circularis*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000311	*Athalia circularis*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000312	*Athalia circularis*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000313	*Athalia lugens*	Female	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000315	*Athalia cordata*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000322	*Athalia lugens*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000323	*Athalia cordata*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000328	*Athalia circularis*	Male	Olsen, K. M.	30.VI-15.VIII.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000331	*Pachyprotasis rapae*	Female	Lønnve, O. J.	13.VI.2011	60.309	10.455	Velotjernet W	Oppland	Norway
SYMVE000333	*Athalia liberta*	Female	Lønnve, O. J.	2.VII-13.VIII.2009	59.51	10.40	Nesodden	Akershus	Norway
SYMVE000335	*Athalia cordata*	Female	Lønnve, O. J.	2.VII-13.VIII.2009	59.49	10.41	Røer	Akershus	Norway
SYMVE000336	*Athalia cordata*	Female	Lønnve, O. J.	2.VII-13.VIII.2009	59.49	10.41	Røer	Akershus	Norway
SYMVE000337	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000338	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000339	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000340	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000341	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway

SYMVE000342	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000343	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000344	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000345	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000346	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000347	*Athalia circularis*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000352	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000353	*Athalia circularis*	Male	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000354	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000355	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000356	*Athalia circularis*	Male	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000357	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000358	*Athalia circularis*	Male	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000359	*Athalia circularis*	Male	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000361	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000362	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000363	*Athalia circularis*	Male	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000364	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000366	*Athalia circularis*	Male	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000369	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000370	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000371	*Athalia circularis*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000372	*Athalia circularis*	Male	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000384	*Athalia circularis*	Male	Lønnve, O. J.	31.V.2005	59.846	10.666	Skoklefall	Akershus	Norway
SYMVE000385	*Pachyprotasis rapae*	Female	Kristiansen, K. B.	19.VI.2012	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000387	*Pachyprotasis rapae*	Female	Kristiansen, K. B.	18.VI.2012	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000388	*Pachyprotasis rapae*	Female	Kristiansen, K. B.	26.VI.2012	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000389	*Pachyprotasis rapae*	Male	Kristiansen, K. B.	13.VI.2012	60.009	10.618	Nordre solberg	Oslo	Norway
SYMVE000390	*Pachyprotasis rapae*	Female	Kristiansen, K. B.	13.VI.2012	60.009	10.618	Nordre solberg	Oslo	Norway

SYMVE000391	*Pachyprotasis rapae*	Female	Lønnve, O. J.	1.VI.2011	59.904	11.139	Jushaugsand	Akershus	Norway
SYMVE000392	*Pachyprotasis rapae*	Male	Kristiansen, K. B.	31.V.2012	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000393	*Pachyprotasis rapae*	Male	Kristiansen, K. B.	31.V.2012	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000394	*Pachyprotasis rapae*	Female	Lønnve, O. J.	1.VI.2011	59.904	11.139	Jushaugsand	Akershus	Norway
SYMVE000395	*Pachyprotasis rapae*	Female	Lønnve, O. J.	1.VI.2011	59.904	11.139	Jushaugsand	Akershus	Norway
SYMVE000396	*Pachyprotasis rapae*	Female	Lønnve, O. J.	1.VI.2011	59.904	11.139	Jushaugsand	Akershus	Norway
SYMVE000397	*Pachyprotasis rapae*	Male	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000398	*Pachyprotasis rapae*	Female	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000399	*Pachyprotasis rapae*	Male	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000400	*Athalia circularis*	Female	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000401	*Pachyprotasis rapae*	Female	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000402	*Pachyprotasis rapae*	Male	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000403	*Athalia circularis*	Male	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000404	*Athalia circularis*	Male	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000405	*Pachyprotasis rapae*	Male	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sonja	Akershus	Norway
SYMVE000454	*Pachyprotasis variegata*	Female	Kristiansen, K. B.	19.VI.2012	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000460	*Pachyprotasis variegata*	Male	Kristiansen, K. B.	31.V.2012	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000461	*Pachyprotasis variegata*	Male	Kristiansen, K. B.	31.V.2012	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000462	*Pachyprotasis variegata*	Male	Kristiansen, K. B.	31.V.2012	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000467	*Pachyprotasis antennata*	Male	Hansen, L. O.	11-26.VI.2007	60.231	11.125	Sessvollmoen W	Akershus	Norway
SYMVE000503	*Athalia rosae*	Female	Lønnve, O. J.	17.VIII.2008	59.193	10.792	Merrapanna	Østfold	Norway
SYMVE000504	*Athalia rosae*	Female	Lønnve, O. J.	17.VIII.2008	59.193	10.792	Merrapanna	Østfold	Norway
SYMVE000505	*Athalia rosae*	Male	Lønnve, O. J.	17.VIII.2008	59.193	10.792	Merrapanna	Østfold	Norway
SYMVE000506	*Athalia rosae*	Male	Lønnve, O. J.	17.VIII.2008	59.193	10.792	Merrapanna	Østfold	Norway
SYMVE000582	*Athalia circularis*	Female	Olsen, T. J.	1.VIII.2008	59.198	11.296	Vest torp	Østfold	Norway
SYMVE000583	*Athalia circularis*	Female	Olsen, T. J.	1.VIII.2008	59.198	11.296	Vest torp	Østfold	Norway
SYMVE000584	*Athalia circularis*	Female	Olsen, T. J.	1.VIII.2008	59.198	11.296	Vest torp	Østfold	Norway
SYMVE000585	*Athalia circularis*	Male	Lønnve, O. J.	17.VIII.2008	59.193	10.792	Merrapanna	Østfold	Norway
SYMVE000586	*Athalia circularis*	Female	Lønnve, O. J.	17.VIII.2008	59.193	10.792	Merrapanna	Østfold	Norway
SYMVE000587	*Athalia circularis*	Male	Lønnve, O. J.	17.VIII.2008	59.193	10.792	Merrapanna	Østfold	Norway

SYMVE000588	*Athalia circularis*	Male	Lønnve, O. J.	17.VIII.2008	59.193	10.792	Merrapanna	Østfold	Norway
SYMVE000589	*Athalia circularis*	Female	Lønnve, O. J.	1.VII- 5.VIII.2012	59.203	10.81	Ødegård, Onsøy	Østfold	Norway
SYMVE000590	*Athalia circularis*	Female	Lønnve, O. J.	1.VII- 5.VIII.2012	59.203	10.81	Ødegård, Onsøy	Østfold	Norway
SYMVE000591	*Pachyprotasis rapae*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000592	*Pachyprotasis rapae*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000593	*Pachyprotasis rapae*	Female	BioFokus (SO)	13.V-11.VII.2009	58.874	9.399	Frydenborgstjenna	Telemark	Norway
SYMVE000594	*Pachyprotasis rapae*	Female	Sand, K.	16.V-14.VI.2004	60.203	12.434	Åranstorpet	Hedemark	Norway
SYMVE000595	*Pachyprotasis rapae*	Male	Hansen, L. O.	5.VI-16.X	60.012	10.787	Dausjøen	Oslo	Norway
SYMVE000596	*Pachyprotasis rapae*	Female	Hansen, L. O.	5.VI-16.X	60.012	10.787	Dausjøen	Oslo	Norway
SYMVE000597	*Pachyprotasis rapae*	Female	Hansen, L. O.	5.VI-16.X	60.012	10.787	Dausjøen	Oslo	Norway
SYMVE000598	*Pachyprotasis rapae*	Male	Hansen, L. O.	5.VI-16.X	60.012	10.787	Dausjøen	Oslo	Norway
SYMVE000599	*Athalia circularis*	Male	Olsen, T. J.	25.V-7.VI.2008	59.198	11.296	Vest torp	Østfold	Norway
SYMVE000600	*Athalia circularis*	Female	Olsen, T. J.	25.V-7.VI.2008	59.198	11.296	Vest torp	Østfold	Norway
SYMVE000601	*Athalia circularis*	Female	Olsen, T. J.	25.V-7.VI.2008	59.198	11.296	Vest torp	Østfold	Norway
SYMVE000602	*Athalia circularis*	Female	Olsen, T. J.	25.V-7.VI.2008	59.198	11.296	Vest torp	Østfold	Norway
SYMVE000603	*Athalia circularis*	Female	Olsen, T. J.	25.V-7.VI.2008	59.198	11.296	Vest torp	Østfold	Norway
SYMVE000604	*Pachyprotasis rapae*	Female	Lønnve, O. J.	12.VI.2010	60.121	10.824	Hakadal verk	Akershus	Norway
SYMVE000605	*Pachyprotasis rapae*	Female	Gammelmo, Ø.	9.VI2006	59.915	10.672	Bygdøy	Oslo	Norway
SYMVE000606	*Athalia circularis*	Female	Olsen, K. M.	13.VII-9.VIII.2007	59.996	10.759	Kirkerud	Akershus	Norway
SYMVE000607	*Athalia circularis*	Female	Olsen, K. M.	13.VII-9.VIII.2007	59.996	10.759	Kirkerud	Akershus	Norway
SYMVE000608	*Athalia circularis*	Female	Olsen, K. M.	13.VII-9.VIII.2007	59.996	10.759	Kirkerud	Akershus	Norway
SYMVE000609	*Athalia circularis*	Male	Olsen, K. M.	13.VII-9.VIII.2007	59.996	10.759	Kirkerud	Akershus	Norway
SYMVE000610	*Athalia circularis*	Male	Olsen, K. M.	13.VII-9.VIII.2007	59.996	10.759	Kirkerud	Akershus	Norway
SYMVE000611	*Athalia circularis*	Male	Olsen, K. M.	13.VII-9.VIII.2007	59.996	10.759	Kirkerud	Akershus	Norway
SYMVE000612	*Pachyprotasis rapae*	Male	Gammelmo, Ø.; Lønnve, O. J.	5.V -3.VII.2012	61.421	8.904	Veslkvelven S	Oppland	Norway
SYMVE000613	*Pachyprotasis rapae*	Female	Gammelmo, Ø.; Lønnve, O. J.	5.V -3.VII.2012	61.421	8.904	Veslkvelven S	Oppland	Norway
SYMVE000614	*Pachyprotasis rapae*	Male	Lønnve, O. J.	22.V.2012	55.564	13.854	Fyledalen	Skåne	Sweden
SYMVE000615	*Pachyprotasis rapae*	Male	Lønnve, O. J.	9.VI.2012	56.788	-3.826	Glen Fender Meadows	Perth and Kinross	Scotland
SYMVE000616	*Pachyprotasis rapae*	Male	Gammelmo, Ø.; Lønnve, O. J.	5.VI-3.VII.2012	61.424	8.886	Storkvolven Ø	Oppland	Norway

SYMVE000617	*Pachyprotasis rapae*	Female	Gammelmo, Ø.; Lønnve, O. J.	5.VI-3.VII.2012	61.424	8.886	Storkvolven Ø	Oppland	Norway
SYMVE000618	*Pachyprotasis rapae*	Male	Lønnve, O. J.	8.VI.2012	68.350	18.832	Abisko	Norrbottens	Sweden
SYMVE000619	*Pachyprotasis rapae*	Male	Lønnve, O. J.	8.VI.2012	68.350	18.832	Abisko	Norrbottens	Sweden
SYMVE000620	*Pachyprotasis rapae*	Female	Lønnve, O. J.	8.VI.2012	68.350	18.832	Abisko	Norrbottens	Sweden
SYMVE000623	*Pachyprotasis rapae*	Female	Hansen, L. O.	25.VI-25.VIII.2007	60.231	11.116	Aurtjernet W, Sessvollmoen	Akershus	Norway
SYMVE000624	*Pachyprotasis rapae*	Female	Hansen, L. O.	25.VI-25.VIII.2007	60.231	11.116	Aurtjernet W, Seeevollmoen	Akershus	Norway
SYMVE000625	*Pachyprotasis rapae*	Female	Hansen, L. O.	25.VI-25.VIII.2007	60.231	11.116	Aurtjernet W, Sessvollmoen	Akershus	Norway
SYMVE000626	*Pachyprotasis rapae*	Female	Hansen, L. O.; Aarvik, L.; Grøndahl, F. A.	25.VI-1.VII.2009	60.807	10.179	Odensberga	Oppland	Norway
SYMVE000628	*Pachyprotasis rapae*	Female	Hansen, L. O.; Grønndahl, F. A.	25.VI-29.VII.2009	60.802	10.128	Dokkadeltaet, Bergsrønningen	Oppland	Norway
SYMVE000629	*Pachyprotasis rapae*	Female	Hansen, L. O.; Grønndahl, F. A.	25.VI-29.VII.2009	60.802	10.128	Dokkadeltaet, Bergsrønningen	Oppland	Norway
SYMVE000630	*Pachyprotasis rapae*	Female	Hansen, L. O.; Grønndahl, F. A.	25.VI-29.VII.2009	60.802	10.128	Dokkadeltaet, Bergsrønningen	Oppland	Norway
SYMVE000631	*Pachyprotasis rapae*	Female	Hansen, L. O.; Grønndahl, F. A.	25.VI-29.VII.2009	60.802	10.128	Dokkadeltaet, Bergsrønningen	Oppland	Norway
SYMVE000632	*Pachyprotasis rapae*	Female	Hansen, L. O.; Grønndahl, F. A.	25.VI-29.VII.2009	60.802	10.128	Dokkadeltaet, Bergsrønningen	Oppland	Norway
SYMVE000633	*Pachyprotasis rapae*	Female	Hansen, L. O.; Grønndahl, F. A.	25.VI-29.VII.2009	60.802	10.128	Dokkadeltaet, Bergsrønningen	Oppland	Norway
SYMVE000634	*Pachyprotasis rapae*	Female	Hansen, L. O.; Grønndahl, F. A.	25.VI-29.VII.2009	60.802	10.128	Dokkadeltaet, Bergsrønningen	Oppland	Norway
SYMVE000635	*Pachyprotasis rapae*	Female	Gammelmo, Ø.	20.VI-25.VII.2006	60.258	10.619	Ristirauvfjellet	Oppland	Norway
SYMVE000636	*Pachyprotasis rapae*	Male	Endrestøl, A.; Hansen, L.O.	29.V-26.VI.2007	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000637	*Pachyprotasis rapae*	Male	Sund, K.	28.V-22.VI.2005	60.196	12.364	Dragonmoen	Hedemark	Norway
SYMVE000638	*Pachyprotasis rapae*	Female	Sagvolden, Svendsen.	5-10.VII.2006	73.903	-29.679	Masi	Finnmark	Norway
SYMVE000645	*Pachyprotasis rapae*	Female	Kristiansen, K. B.	28.VII.2006	59.996	10.759	Kirkeby	Oslo	Norway
SYMVE000646	*Pachyprotasis rapae*	Female	Reiso, S.	13.VI-13.VII.2009	59.990	8.820	Tinn	Telemark	Norway
SYMVE000647	*Pachyprotasis rapae*	Male	Olberg, S.	30.IV-19.V.2008	52.147	7.732	Slagentagen	Vestfold	Norway
SYMVE000648	*Pachyprotasis rapae*	Female	Lønnve, O. J.	1.VI.2011	59.904	11.139	Jushaugsand	Akershus	Norway
SYMVE000649	*Pachyprotasis rapae*	Female	Olsen, T, J.	1-26.VI.2009	58.889	11.540	Grefsrød	Østfold	Norway

SYMVE000650	*Pachyprotasis rapae*	Female	Olsen, K. M.	19.V-30.VI.2011	59.966	11.032	Sogna	Akershus	Norway
SYMVE000651	*Pachyprotasis rapae*	Male	Lønnve, O. J.	2.VI.2008	59.581	12.096	Silvatnet	Hedemark	Norway
SYMVE000652	*Pachyprotasis rapae*	Female	Olberg, S.	30.V.2012	60.077	10.891	Berg sandtak	Akershus	Norway
SYMVE000653	*Pachyprotasis rapae*	Female	Olsen, K. M.	29.V-30.VI	59.935	10.822	Risløkkafartet	Oslo	Norway
SYMVE000654	*Pachyprotasis rapae*	Female	Olsen, K. M.	29 .V.2010	61.064	11.36	Bjørnstadøya	Hedemark	Norway
SYMVE000655	*Pachyprotasis rapae*	Female	Sund, K.	16.V-14.VI.2004	60.203	12.434	Åranstorpet	Hedemark	Norway

Table 2-A. Information about the investigated specimens of *Athalia circularis* and *Pachyprotasis rapae*, overview of the morphology scores, COI haplogroups, and local population (based on the EIS-system) used for statistical analysis of COI and morphology, respectively.

Sample	Species	Sex	Mesepisternum colouration	Ventral colouration	Tergites colouration	Haplogroup	Population (AMOVA)	Population (morphology)
SYMVE000029	*Athalia circularis*	Female	2	1	-	2	3	3
SYMVE000103	*Athalia circularis*	Female	2	1	-	1	3	3
SYMVE000150	*Athalia circularis*	Female	1	1	-	1	6	6
SYMVE000151	*Athalia circularis*	Female	3	2	-	1	6	6
SYMVE000178	*Athalia circularis*	Female	2	1	-	2	3	3
SYMVE000180	*Athalia circularis*	Female	4	2	-	1	3	3
SYMVE000197	*Athalia circularis*	Male	2	2	-	1	6	6
SYMVE000210	*Athalia circularis*	Male	2	2	-	2	5	5
SYMVE000221	*Athalia circularis*	Female	4	2	-	1	6	6
SYMVE000222	*Athalia circularis*	Female	2	1	-	1	6	6
SYMVE000295	*Athalia circularis*	Female	4	2	-	-	-	3
SYMVE000296	*Athalia circularis*	Female	4	2	-	1	3	3
SYMVE000297	*Athalia circularis*	Male	3	2	-	1	3	3
SYMVE000302	*Athalia circularis*	Male	3	2	-	2	1	1
SYMVE000303	*Athalia circularis*	Female	4	2	-	1	4	4
SYMVE000304	*Athalia circularis*	Female	4	2	-	1	4	4
SYMVE000305	*Athalia circularis*	Male	2	2	-	1	4	4
SYMVE000306	*Athalia circularis*	Male	3	2	-	1	4	4
SYMVE000307	*Athalia circularis*	Male	1	2	-	1	4	4
SYMVE000308	*Athalia circularis*	Male	3	2	-	-	-	4
SYMVE000309	*Athalia circularis*	Male	3	2	-	1	4	4
SYMVE000310	*Athalia circularis*	Male	2	2	-	1	4	4
SYMVE000311	*Athalia circularis*	Male	3	2	-	1	4	4
SYMVE000312	*Athalia circularis*	Male	1	2	-	2	4	4
SYMVE000328	*Athalia circularis*	Male	3	2	-	1	4	4
SYMVE000337	*Athalia circularis*	Female	1	1	-	2	5	5

SYMVE000338	*Athalia circularis*	Female	1	1	-	1	5	5
SYMVE000339	*Athalia circularis*	Female	1	2	-	1	5	5
SYMVE000340	*Athalia circularis*	Female	1	1	-	2	5	5
SYMVE000341	*Athalia circularis*	Female	1	1	-	1	5	5
SYMVE000342	*Athalia circularis*	Female	2	1	-	1	5	5
SYMVE000343	*Athalia circularis*	Female	2	1	-	1	5	5
SYMVE000344	*Athalia circularis*	Female	1	1	-	-	-	5
SYMVE000345	*Athalia circularis*	Female	1	1	-	2	5	5
SYMVE000346	*Athalia circularis*	Female	2	1	-	1	5	5
SYMVE000347	*Athalia circularis*	Female	1	1	-	2	5	5
SYMVE000352	*Athalia circularis*	Female	3	2	-	2	-	1
SYMVE000353	*Athalia circularis*	Male	3	2	-	2	1	1
SYMVE000354	*Athalia circularis*	Female	3	2	-	1	1	1
SYMVE000355	*Athalia circularis*	Female	2	1	-	1	1	1
SYMVE000356	*Athalia circularis*	Male	1	1	-	1	1	1
SYMVE000357	*Athalia circularis*	Female	4	2	-	1	1	1
SYMVE000358	*Athalia circularis*	Male	2	2	-	2	1	1
SYMVE000359	*Athalia circularis*	Male	2	1	-	1	1	1
SYMVE000361	*Athalia circularis*	Female	4	2	-	2	1	1
SYMVE000362	*Athalia circularis*	Female	3	2	-	2	1	1
SYMVE000363	*Athalia circularis*	Male	3	2	-	1	1	1
SYMVE000364	*Athalia circularis*	Female	2	1	-	1	1	1
SYMVE000366	*Athalia circularis*	Male	3	2	-	2	1	1
SYMVE000369	*Athalia circularis*	Female	3	2	-	1	1	1
SYMVE000370	*Athalia circularis*	Female	1	1	-	2	1	1
SYMVE000371	*Athalia circularis*	Female	1	1	-	2	1	1
SYMVE000372	*Athalia circularis*	Male	1	1	-	1	1	1
SYMVE000384	*Athalia circularis*	Male	3	1	-	1	3	3
SYMVE000400	*Athalia circularis*	Female	2	1	-	1	4	4

SYMVE000403	*Athalia circularis*	Male	3	2	-	1	4	4
SYMVE000404	*Athalia circularis*	Male	4	2	-	1	4	4
SYMVE000582	*Athalia circularis*	Female	4	2	-	1	2	2
SYMVE000583	*Athalia circularis*	Female	4	2	-	1	2	2
SYMVE000584	*Athalia circularis*	Female	3	2	-	1	2	2
SYMVE000585	*Athalia circularis*	Male	4	2	-	1	2	2
SYMVE000586	*Athalia circularis*	Female	4	2	-	1	2	2
SYMVE000587	*Athalia circularis*	Male	4	2	-	1	2	2
SYMVE000588	*Athalia circularis*	Male	4	2	-	1	2	2
SYMVE000589	*Athalia circularis*	Female	4	2	-	2	2	2
SYMVE000590	*Athalia circularis*	Female	4	2	-	1	2	2
SYMVE000599	*Athalia circularis*	Male	1	2	-	1	2	2
SYMVE000600	*Athalia circularis*	Female	2	2	-	1	2	2
SYMVE000601	*Athalia circularis*	Female	4	2	-	-	2	2
SYMVE000602	*Athalia circularis*	Female	4	2	-	2	2	2
SYMVE000603	*Athalia circularis*	Female	4	2	-	1	2	2
SYMVE000606	*Athalia circularis*	Female	4	2	-	1	3	3
SYMVE000607	*Athalia circularis*	Female	3	2	-	1	3	3
SYMVE000608	*Athalia circularis*	Female	3	2	-	1	3	3
SYMVE000609	*Athalia circularis*	Male	3	1	-	1	3	3
SYMVE000610	*Athalia circularis*	Male	2	2	-	-	-	3
SYMVE000611	*Athalia circularis*	Male	1	2	-	-	-	3
SYMVE000040	*Pachyprotasis rapae*	Female	3	3	3	2	2	2b
SYMVE000045	*Pachyprotasis rapae*	Female	2	2	2	1	2	2b
SYMVE000140	*Pachyprotasis rapae*	Male	2	2	1	1	4	4
SYMVE000154	*Pachyprotasis rapae*	Male	1	1	1	1	1	1
SYMVE000209	*Pachyprotasis rapae*	Female	3	1	2	2	2	2b
SYMVE000250	*Pachyprotasis rapae*	Male	1	2	1	1	2	2b
SYMVE000294	*Pachyprotasis rapae*	Female	3	3	2	2	-	3
SYMVE000331	*Pachyprotasis rapae*	Female	1	2	2	-	-	1

SYMVE000385	*Pachyprotasis rapae*	Female	3	3	2	-	-	1
SYMVE000387	*Pachyprotasis rapae*	Female	2	3	3	1	1	1
SYMVE000388	*Pachyprotasis rapae*	Female	2	3	1	1	1	1
SYMVE000389	*Pachyprotasis rapae*	Male	3	2	3	2	1	1
SYMVE000390	*Pachyprotasis rapae*	Female	2	3	2	2	1	1
SYMVE000391	*Pachyprotasis rapae*	Female	2	3	2	-	-	2a
SYMVE000392	*Pachyprotasis rapae*	Male	1	3	1	-	-	1
SYMVE000393	*Pachyprotasis rapae*	Male	1	2	1	2	-	1
SYMVE000394	*Pachyprotasis rapae*	Female	3	3	2	-	1	2a
SYMVE000395	*Pachyprotasis rapae*	Female	2	3	3	-	-	2a
SYMVE000396	*Pachyprotasis rapae*	Female	2	3	3	1	2	2a
SYMVE000397	*Pachyprotasis rapae*	Male	2	3	1	2	2	2a
SYMVE000398	*Pachyprotasis rapae*	Female	3	3	3	-	-	2a
SYMVE000399	*Pachyprotasis rapae*	Male	1	3	1	2	2	2a
SYMVE000401	*Pachyprotasis rapae*	Female	3	3	1	-	-	2a
SYMVE000402	*Pachyprotasis rapae*	Male	1	2	1	1	2	2a
SYMVE000405	*Pachyprotasis rapae*	Male	2	3	1	2	2	2a
SYMVE000591	*Pachyprotasis rapae*	Female	3	3	3	-	-	-
SYMVE000592	*Pachyprotasis rapae*	Female	1	2	2	-	-	-
SYMVE000593	*Pachyprotasis rapae*	Female	2	3	3	2	-	-
SYMVE000594	*Pachyprotasis rapae*	Female	3	1	3	-	-	4
SYMVE000595	*Pachyprotasis rapae*	Male	2	3	1	-	-	1
SYMVE000596	*Pachyprotasis rapae*	Female	3	3	2	-	-	1
SYMVE000597	*Pachyprotasis rapae*	Female	2	3	3	1	1	1
SYMVE000598	*Pachyprotasis rapae*	Male	2	3	1	1	1	1
SYMVE000604	*Pachyprotasis rapae*	Female	3	3	3	-	-	1
SYMVE000605	*Pachyprotasis rapae*	Female	2	3	3	-	-	2b
SYMVE000612	*Pachyprotasis rapae*	Male	1	2	3	-	-	3
SYMVE000613	*Pachyprotasis rapae*	Female	1	3	1	2	3	3

SYMVE000614	*Pachyprotasis rapae*	Male	1	2	1	1	-	-
SYMVE000615	*Pachyprotasis rapae*	Male	2	3	1	1	-	-
SYMVE000616	*Pachyprotasis rapae*	Male	1	2	1	2	3	3
SYMVE000617	*Pachyprotasis rapae*	Female	3	3	2	-	-	3
SYMVE000618	*Pachyprotasis rapae*	Male	2	3	1	-	-	-
SYMVE000619	*Pachyprotasis rapae*	Male	3	3	3	-	-	-
SYMVE000620	*Pachyprotasis rapae*	Female	3	3	2	-	-	-
SYMVE000623	*Pachyprotasis rapae*	Female	2	3	3	-	-	4
SYMVE000624	*Pachyprotasis rapae*	Female	1	3	2	1	4	4
SYMVE000625	*Pachyprotasis rapae*	Female	2	3	2	2	4	4
SYMVE000626	*Pachyprotasis rapae*	Female	1	3	2	2	3	3
SYMVE000628	*Pachyprotasis rapae*	Female	2	3	1	1	3	3
SYMVE000629	*Pachyprotasis rapae*	Female	2	3	1	-	-	3
SYMVE000630	*Pachyprotasis rapae*	Female	2	3	2	1	3	3
SYMVE000631	*Pachyprotasis rapae*	Female	3	3	1	-	-	3
SYMVE000632	*Pachyprotasis rapae*	Female	2	3	1	-	-	3
SYMVE000633	*Pachyprotasis rapae*	Female	2	3	2	2	3	3
SYMVE000634	*Pachyprotasis rapae*	Female	2	3	1	-	-	3
SYMVE000635	*Pachyprotasis rapae*	Female	2	3	1	1	1	1
SYMVE000636	*Pachyprotasis rapae*	Male	3	3	1	-	-	1
SYMVE000637	*Pachyprotasis rapae*	Male	2	3	1	-	-	4
SYMVE000638	*Pachyprotasis rapae*	Female	1	3	1	-	-	-
SYMVE000645	*Pachyprotasis rapae*	Female	1	3	1	1	1	1
SYMVE000647	*Pachyprotasis rapae*	Male	1	2	1	-	-	2b
SYMVE000648	*Pachyprotasis rapae*	Female	2	3	1	1	2	2a
SYMVE000649	*Pachyprotasis rapae*	Female	1	3	1	-	-	2a
SYMVE000650	*Pachyprotasis rapae*	Female	1	3	1	1	2	2a
SYMVE000651	*Pachyprotasis rapae*	Male	2	3	1	-	-	4
SYMVE000652	*Pachyprotasis rapae*	Female	3	3	1	2	1	1
SYMVE000653	*Pachyprotasis rapae*	Female	3	3	3	-	-	2b

SYMVE000654	*Pachyprotasis rapae*	Female	3	3	2	1	4	4
SYMVE000655	*Pachyprotasis rapae*	Female	3	3	1	-	-	4

Appendix 2

Table 3-A. Independent-samples t-test on morphology differences between the two COI haplotypes of *Pachyprotasis rapae*.

	Mesepisternum colouration	Ventral colouration	Tergites colouration
t	-1.299	-0.382	-0.670
df	36	36	36
Sig. (2-tailed)	0.202	0.704	0.507
Mean Difference	-0.300	-0.072	-0.172
Std. Error Difference	0.231	0.189	0.257
95 % CI lower limit	-0.769	-0.455	-0.694
95 % CI upper limit	0.169	0.311	0.349

Table 4-A. ANOVA of body morphology in local populations of *Pachyprotasis rapae* (populations defined based on the European invertebrate survey (EIS)-system; see Table 4 for details).

	Sum of squares	df	Mean square	F	Sig.
Mesepisternum colouration					
Between groups	2.078	4	0.520	1.825	0.137
Within groups	15.655	55	0.285		
Total	17.733	59			
Ventral colouration					
Between groups	0.730	4	0.183	0.302	0.875
Within groups	33.203	55	0.604		
Total	33.933	59			
Tergites colouration					
Between groups	1.880	4	0.470	0.704	0.593
Within groups	36.720	55	0.668		
Total	38.600	59			

Table 5-A. Independent-samples t-test on body morphology differences between the two COI haplogroups of *Athalia circularis*.

	Mesepisternum colouration	Ventral colouration
t	1.858	0.886
df	69	69
Sig. (2-tailed)	0.067	0.379
Mean Difference	0.539	0.113
Std. Error Difference	0.290	0.128
95 % CI lower limit	-0.040	-0.142
95 % CI upper limit	1.119	0.369

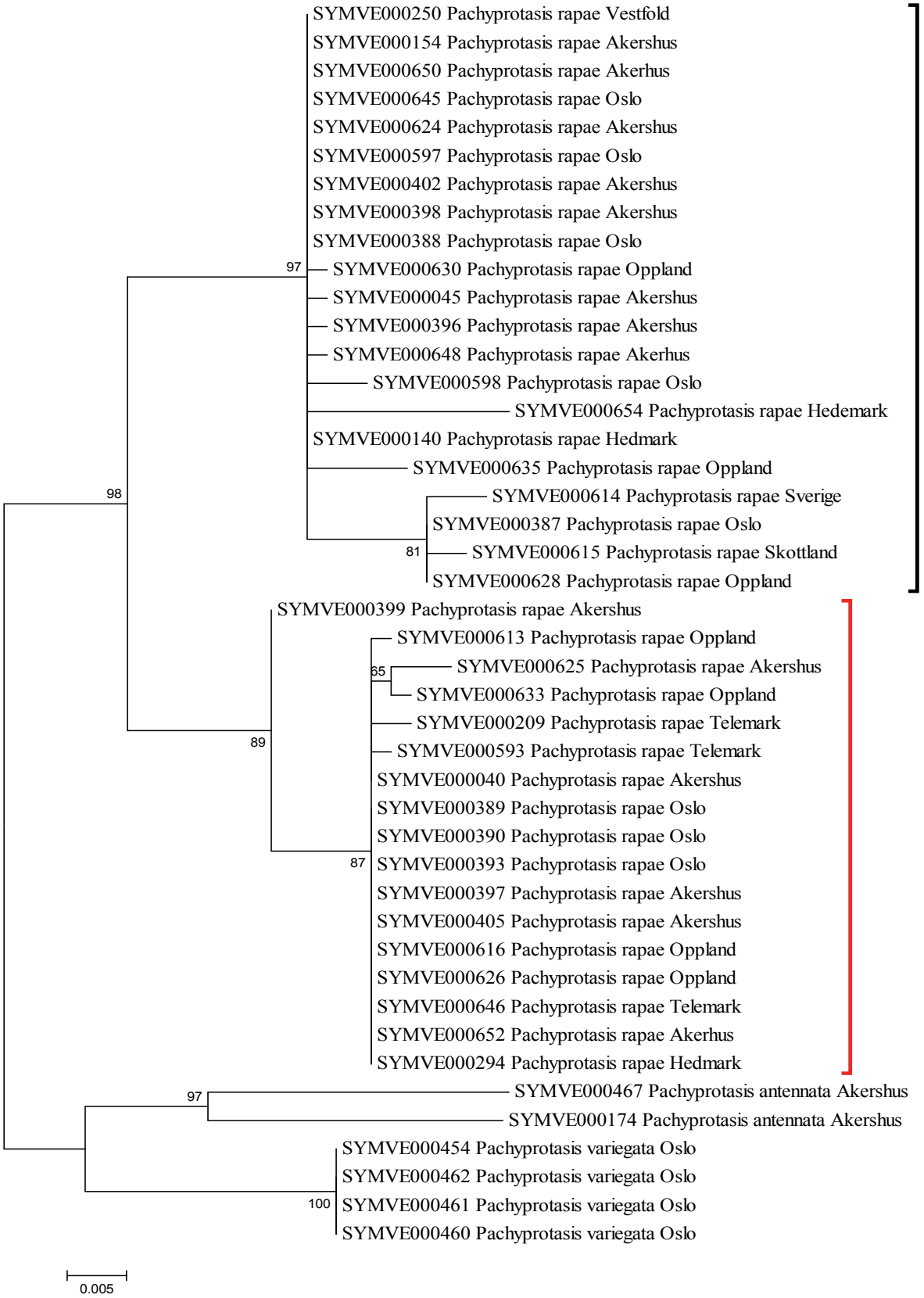

Figure 1-A. Maximum Likelihood analysis performed on 44 COI sequences of *Pachyprotasis* with 1000 bootstrap replicates (only support values above 75 % shown). *P. rapae* haplogroup 1 = black; haplogroup 2 = red.

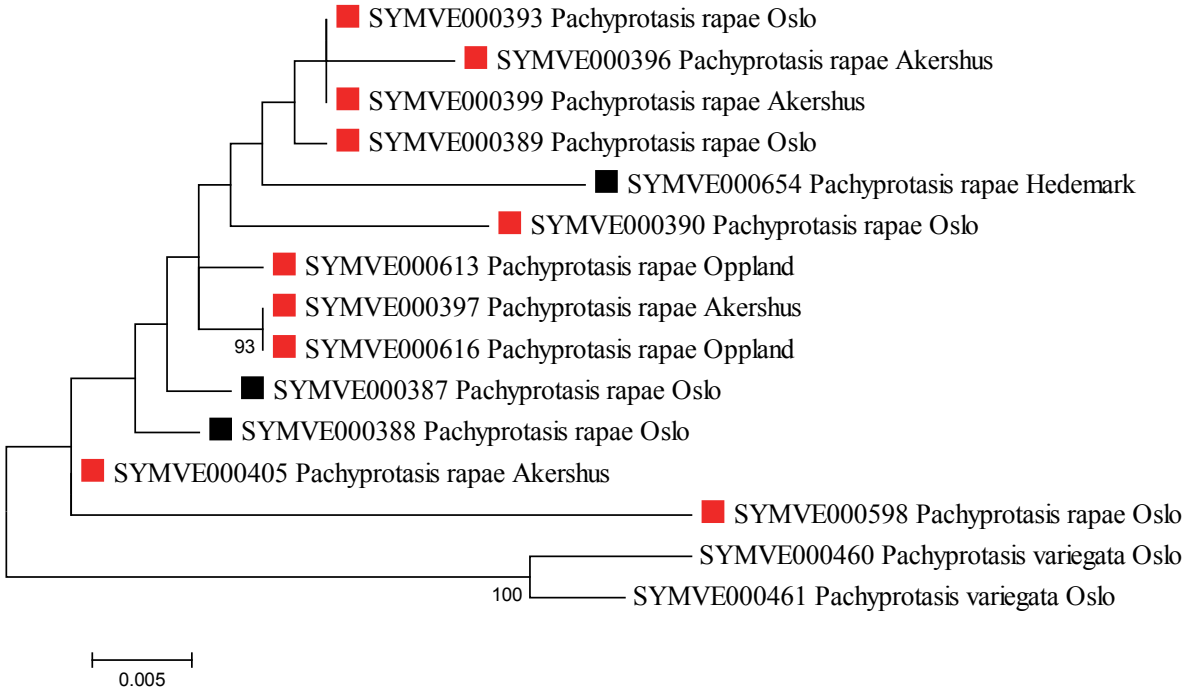

Figure 2-A. Maximum Likelihood analysis performed on 15 CAD sequences of *Pachyprotasis* with 500 bootstrap replicates (only support values above 75 % shown). *P. rapae* specimens *with* COI haplogroup 1 = black squares. *P. rapae* specimens with COI haplogroup 2 = red squares.

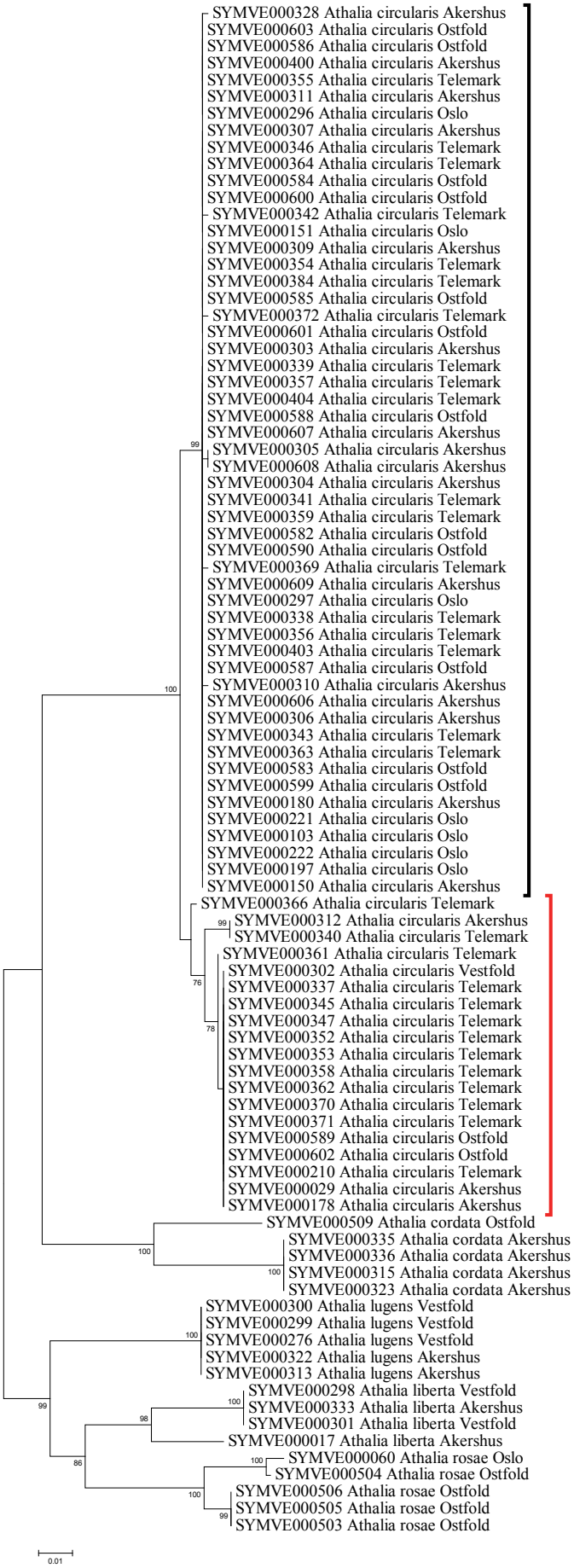

Figure 3-A. Neighbour-joining analysis performed on 91 COI sequences of *Athalia* with 1000 bootstrap replicates (only support values above 75 % shown). *A. circularis* haplogroup 1 = black; haplogroup 2 = red. All four out-group taxa are included in the analysis.

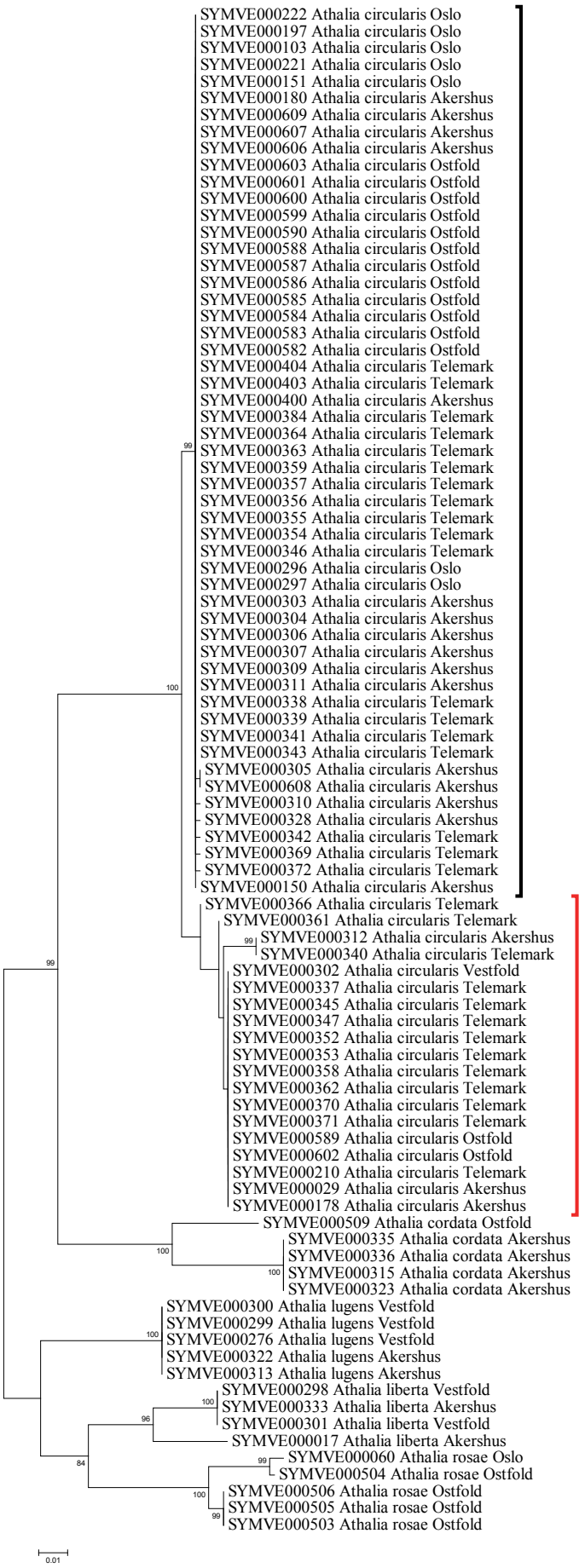

Figure 4-A. Maximum Likelihood analysis performed on 91 COI sequences of *Athalia* with 1000 bootstrap replicates (only support values above 75 % shown). *A. circularis* haplogroup 1 = black; haplogroup 2 = red. All four out-group taxa are included in the analysis.

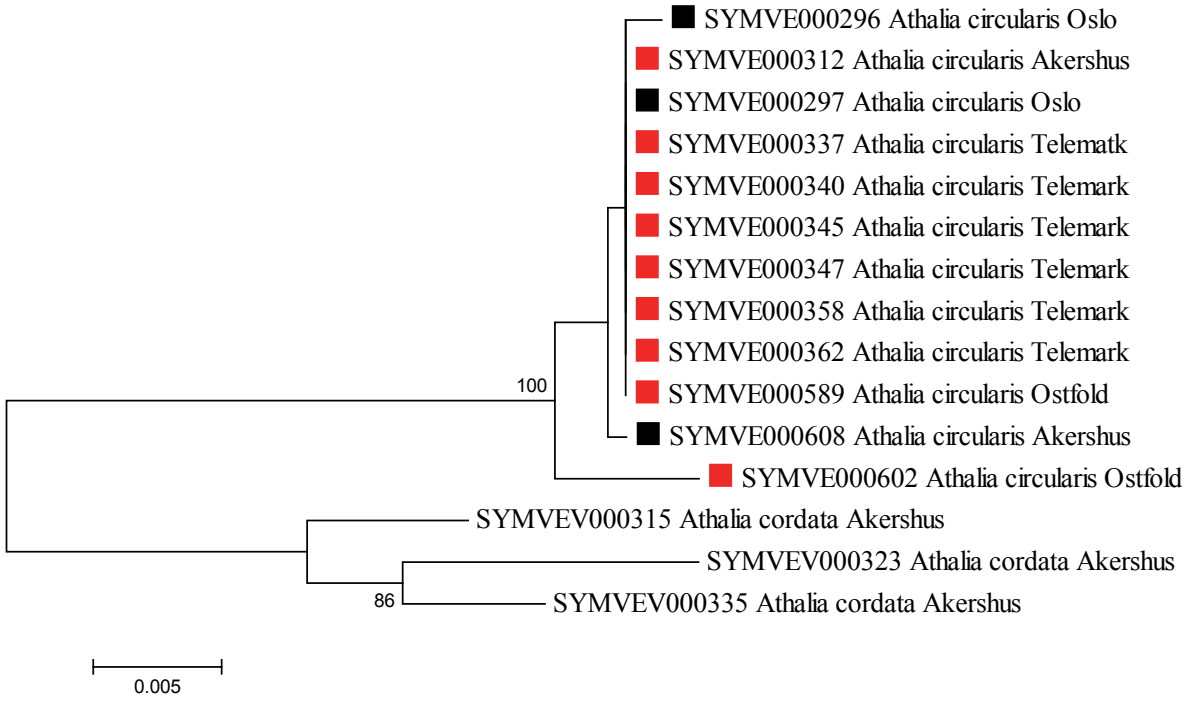

Figure 5-A. Maximum Likelihood analysis performed on 15 CAD sequences of *Athalia* with 500 bootstrap replicates (only support values above 75 % shown). *A. circularis* specimens with COI haplogroup 1 = black squares. *A. circularis* specimens with COI haplogroup 2 = red squares.

Appendix 3

Detailed description of female genitalia for selected specimens.

Pachyprotasis

P. variegata (out-group)

The saw of ***P. variegata*** (Sym. 454) has a total of 19 denticles. Each denticle with W-shaped line, except for number 1 and 2.
Denticle 1 with rounded apex, with no W-shape. There are no jagged edges on the posterior side. The anterior side is straighter with 1 tip.
Denticle 2 with rounded apex, with a V-shape. The jagged edges of the posterior side are reduced. The anterior side has a gradual slope with two tips.
Denticles 3-4 with rounded apices. Posterior side with basal half jagged. Anterior end has a gradual slope with 1 tip.
Denticles 5-6 with rounded apices. Posterior side with basal half jagged. Anterior side with gradual slope and one tip.
Denticle 7 with narrow apex, Anterior side is straight with 3 tips.
Denticles 8-10 with narrow apices. Both posterior and anterior sides sharp. The posterior side is jagged on basal half. Anterior side with two tips.
Denticles 11-14 are equally shaped. Posterior side with jagged edges on basal half. Anterior side straighter, with one indent.
Denticles 15-19 are wave-shaped, with bare apex. The posterior side has clear and sharp jagged edges.

P. rapae

Haplogroup 1:

The saw of **Sym. 387** has a total of 16 denticles. Each denticle with W-shaped line, except for number 1.
Denticle 1 with broad round apex, with no W-shape. There are no jagged edges on the posterior side. The anterior side is straighter with 1 tip.
Denticle 2 with broad apex und tip of the denticle and a slight slope on both sides, anterior hump. No jagged edges.
Denticle 3 rounded apex. There are no jagged edges on the posterior side. The anterior side with gradual slope and 2 tips.
Denticles 4-5 with rounded apices. The jagged edges of the posterior side are reduced. Anterior side with gradual slope and two dull tips.
Denticles 6-7 with rounded apices. Posterior side with basal half jagged. Anterior side with gradual slope and hump.
Denticles 8-11 are equally shaped with rounded apices. Posterior side with jagged edges on basal half. Anterior side straighter, with 2 tips.

Denticles 12-13 are wave-shaped, with bare apex. Posterior side with jagged edges on basal half. Anterior side with 1 indent
Denticles 14-15 are wave-shaped, with bare apex. The posterior side has dull jagged edges.

The saw of **Sym. 387** has a total of 17 denticles. Each denticle with W-shaped line, except for number 1-2.
Denticle 1 with broad apex. There are no jagged edges on the posterior side. Anterior side straighter.
Denticle 2 with broad apex. The jagged edges of the posterior side are reduced. Anterior side straighter with 2 dull tips.
Denticles 3-5 with narrow apices. The jagged edges of the posterior side are reduced. Anterior side with gradual slope and hump with 2 dull tips.
Denticle 6 with narrow apex. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 2 tips.
Denticles 7-9 with narrow apices. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 1 tip.
Denticles 10-11 rounded apex. Posterior side with jagged edges on basal half. Anterior side straighter.
Denticle 12 rounded apex. Posterior side with jagged edges on basal half. Anterior side straighter, with 2 tips.
Denticles 13-16 are wave-shaped, with bare apex. Posterior side with jagged edges on basal half. Anterior side with 1 indent.

The saw of **Sym. 36** has a total of 18 denticles. Each denticle with W-shaped line, except for number 1-2.
Denticle 1 with broad apex. There are no jagged edges on the posterior side. Posterior side and anterior side straighter.
Denticle 2 with rounded apex. There are no jagged edges on the posterior side. Anterior side straighter and hump.
Denticle 3 with rounded apex. The jagged edges of the posterior side are reduced. Anterior side with gradual slope and hump with 2 dull tips.
Denticle 4-6 with rounded apex. The jagged edges of the posterior side are reduced. Anterior side with gradual slope and 1 dull tips.
Denticle 7 with rounded apex. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 2 tip.
Denticle 8 with rounded apex. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 1 tip.
Denticles 9-12 with narrow apices. Posterior side with jagged edges on basal half. Anterior side straighter and 2 tips.
Denticles 13-18 are wave-shaped, with bare apex. Posterior side with jagged edges on basal half. Anterior side with 1 indent.

The saw of **Sym. 597** has a total of 16 denticles. Each denticle with W-shaped line, except for number 1-2.

Denticle 1 are wave-shaped, with bare apex. There are no jagged edges on the posterior side. Anterior side with 1 indent.
Denticle 2 with rounded apex. The jagged edges of the posterior side are reduced. The anterior side with gradual slope and hump with 2 tips.
Denticle 3 with rounded apex. The jagged edges of the posterior side are reduced. The anterior side with gradual slope and 2 tips.
Denticle 4 with rounded apex. Posterior side with jagged edges on basal half. The anterior side with gradual slope and hump with 2 tips.
Denticle 5 with narrow apex. Posterior side with jagged edges on basal half. Anterior side with gradual slope and hump with 1 tip.
Denticle 6 broken. Posterior side with jagged edges on basal half.
Denticle 7 with narrow apex. Posterior side with jagged edges on basal half. Anterior side with gradual slope and hump with 2 tips.
Denticle 8 broken.
Denticles 9-11 with narrow apices. Posterior side with jagged edges on basal half. Anterior side straighter and 2 tips.
Denticles 12-14 are wave-shaped, with bare apex. The jagged edges of the posterior side are reduced. Anterior side with 1 indent.
Denticle 15 are wave-shaped, with bare apex. There are no jagged edges on the posterior side.

The saw of **Sym. 645** has a total of 18 denticles. Each denticle with W-shaped line, except for number 1-2.
Denticle 1 with broad apex. There are no jagged edges on the posterior side. Posterior side and anterior side straighter and hump with indent.
Denticle 2 with rounded apex. There are no jagged edges on the posterior side.
The anterior side with gradual slope and hump with 2 tips.
Denticles 3-6 with rounded apex. The jagged edges of the posterior side are reduced.
The anterior side with gradual slope and hump with 2 tips.
Denticle 7 with rounded apex. Posterior side with jagged edges on basal half. The anterior side with gradual slope and with 2 tips.
Denticle eleven not visible.
Denticle 8 with rounded apex. Posterior side with jagged edges on basal half. Rest not visible.
Denticle 9 with rounded apex. Posterior side with jagged edges on basal half.
The anterior side straighter and 2 tips.
Denticle 10 with rounded apices. The jagged edges of the posterior side are reduced.
The anterior side straighter and 2 tips.
Denticles 11-12 with rounded apices. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 2 tips.
Denticles 13-15 are wave-shaped, with bare apex. The jagged edges of the posterior side are reduced. Anterior side with 1 indent.
Denticles 16 are wave-shaped, with bare apex. The jagged edges of the posterior side are reduced.

Haplogroup 2:

The saw of **Sym. 40** has a total of 18 denticles. Each denticle with W-shaped line, except for number 1-2.
Denticle 1 with broad apex. There are no jagged edges on the posterior side. Posterior side and anterior side straight

Denticles 2-5 with narrow apices. The jagged edges of the posterior side are reduced.
The anterior side with gradual slope with hump.
Denticle 6 with narrow apex. The jagged edges of the posterior side are reduced.
The anterior side with gradual slope and 1 tip.
Denticles 7-10 with narrow apices. The jagged edges of the posterior side are reduced.
The anterior side with 2 tips.
Denticle 11 with narrow apex. The jagged edges of the posterior side are reduced.
Denticle 12 with narrow apex. The jagged edges of the posterior side are reduced.
The anterior side with 2 tips.
Denticles 13-14 with narrow apices. The jagged edges of the posterior side are reduced.
The anterior side with indent.
Denticles 15-16 are wave-shaped, with bare apex. The jagged edges of the posterior side are reduced.

The saw of **Sym. 209** has a total of 16 denticles. Each denticle with W-shaped line, except for number 1.
Denticle 1 with broad apex. There are no jagged edges on the posterior side. Posterior side and anterior side straight.
Denticle 2 with rounded apex. There are no jagged edges on the posterior side. The anterior side with gradual slope and hump.
Denticle 3 with rounded apex. The jagged edges of the posterior side are reduced. The anterior side with gradual slope and small hump with 2 tips.
Denticles 4-5 with rounded apex. The jagged edges of the posterior side are reduced. The anterior side with gradual slope and with 2 tips.
Denticles 6-7 with rounded apex. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 2 tips.
Denticle 8 with rounded apex. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 1 tip.
Denticles 9-11 with narrow apices. Posterior side with jagged edges on basal half. Anterior side straighter and 2 tips.
Denticles 12-14 are wave-shaped, with bare apex. The jagged edges of the posterior side are reduced. Anterior side with 1 indent.
Denticle 15 is wave-shaped, with bare apex. There are no jagged edges on the posterior side.

The saw of **Sym. 294** has a total of 16 denticles. Each denticle with W-shaped line, except for number 1-2.
Denticle 1 with broad apex. There are no jagged edges on the posterior side. Posterior side and anterior side straight.
Denticle 2 with rounded apex. There are no jagged edges on the posterior side. The anterior side straighter.
Denticles 3-6 with narrow apices. The jagged edges of the posterior side are reduced. The anterior side with gradual slope and hump.
Denticles 7-9 with rounded apices. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 2 tips.
Denticles 10-12 with narrow apices. Posterior side with jagged edges on basal half. Anterior side straighter and 2 tips.
Denticles 13-15 are wave-shaped, with bare apex. The jagged edges of the posterior side are reduced. Anterior side with 1 indent.

The saw of **Sym. 390** has a total of 18 denticles. Each denticle with W-shaped line, except for number 1-2.
Denticle 1 with broad apex. There are no jagged edges on the posterior side. Posterior side and anterior side straight.
Denticle 2 with rounded apices. There are no jagged edges on the posterior side The anterior side with gradual slope.
Denticle 3 with rounded apices. Posterior side with jagged edges on basal half. The anterior side with gradual slope and hump with 2 tips.
Denticles 4-5 with rounded apices. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 1 tips.
Denticles 6-10 with rounded apices. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 2 tips.
Denticles 11-13 with narrow apices. Posterior side with jagged edges on basal half. Anterior side straighter and 2 tips.
Denticles 14-16 are wave-shaped, with bare apex. Posterior side with jagged edges on basal half. Anterior side with 1 indent.
Denticle 17 are half circle shaped, with bare apex. The jagged edges of the posterior side are reduced.

The saw of **Sym. 613** has a total of 18 denticles. Each denticle with W-shaped line, except for number 1-2.
Denticle 1 with broad apex. There are no jagged edges on the posterior side. Anterior side straight and small hump.
Denticle 2 with broad apex. There are no jagged edges on the posterior side. Anterior side straight and hump.
Denticle 3 with rounded apex. The jagged edges of the posterior side are reduced. The anterior side with gradual slope and hump with indent.
Denticle 4-6 with rounded apices. The jagged edges of the posterior side are reduced. The anterior side with gradual slope and 2 tips.
Denticles 7 with narrow apex. Posterior side with jagged edges on basal half. Anterior side straighter and 3 tips.
Denticles 8 with narrow apex. Posterior side with jagged edges on basal half. Anterior side straighter and 2 tips.
Denticle 9-10 with rounded apices. Posterior side with jagged edges on basal half. The anterior side with gradual slope and 2 tips.
Denticles 11-13 with narrow apices. Posterior side with jagged edges on basal half. Anterior side straighter and 2 tips.
Denticles 14-16 are wave-shaped, with bare apex. Posterior side with jagged edges on basal half. Anterior side with 1 indent.

Athalia

A. cordata (out-group)

The saw of ***A. cordata*** has a total of 15 denticles. All denticles long and pointy. The denticles have a black v or single line shape above it.
Denticle 1 with broad apex. Posterior side gradual slope. Anterior side straight. 2 light lines.
Denticles 2-4 with narrow apex. Posterior side straight with jagged edges. Anterior side straight with jagged edges. 1 long black line
Denticles 5-10 with narrow apex. Posterior side straight. Anterior side straight with jagged edges. Point round tip. 1 long, 1 short black line.
Denticle 11 with narrow apex. Posterior side slight slope. Anterior side straighter with jagged edges. 1 long, 1 short black line.
Denticles 12-13 posterior side slight slope. Anterior side straighter with jagged edges reduced. 1 long, 1 short black line.
Denticle 14 posterior side slight slope. Anterior side straighter end with a hump. 1 black line.

A. circularis

Haplogroup 1:

The saw of **Sym. 342** has a total of 17 denticles. Each denticle with W or V-shaped line.
Denticle 1 very small sized. Posterior side slight slope. Anterior side straighter slope. Two lines down. No jagged edges.
Denticle 2 small sized. Posterior side slight slope. Anterior side slight slope. Two lines down. No jagged edges.
Denticle 3 small sized. Posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 4 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. Two lines down.
Denticles 6-7 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 8 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. Two separated lines down.
Denticles 9-10 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 11-12 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 13 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticle 14 with narrow apex. Posterior side slight slope with jagged edges. Anterior side straighter with jagged edges. W shaped line.
Denticles 15-16 posterior side slight slope. Anterior side straighter end. W shaped line.

The saw of **Sym. 343** has a total of 16 denticles. Each denticle with W or V-shaped line.

Denticle 1 very small sized. Posterior side slight slope. Anterior side straighter slope. Two lines down. No jagged edges.
Denticle 2 small sized. Posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. Two lines down.
Denticle 3 small sized. Posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticles 4-5 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 6-10 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 11 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticle 12 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 13 posterior side slight slope with reduced jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 14-15 posterior side slight slope with jagged edges. Anterior side straighter with jagged edges. W shaped line.
Denticle 16 posterior side slight slope. Anterior side straighter end. W shaped line.

The saw of **Sym. 388** has a total of 17 denticles. Each denticle with W or V-shaped line.
Denticle 1 very small sized. Posterior side very slight slope with reduced jagged edges. Anterior side very slight slope. 2 lines down.
Denticle 2 small sized. Posterior side slight slope with reduced jagged edges. Anterior side slight slope with reduced jagged edges. 2 lines down.
Denticles 3-4 small sized. Posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. 2 lines down.
Denticles 5-9 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 10-11 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticles 12-13 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 14-15 posterior side slight slope with reduced jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticle 16 posterior side slight slope with jagged edges. Anterior side straighter with jagged edges. W shaped line.
Denticle 17 posterior side slight slope. Anterior side straighter end. V shaped line.

The saw of **Sym. 590** has a total of 16 denticles. Each denticle with W or V-shaped line.
Denticle 1 very small sized. Posterior side very slight slope with. Anterior side very slight slope reduced jagged edges. Two lines down.
Denticles 2-7 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 8-11 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 12 posterior side slight slope with reduced jagged edges. Anterior side slight slope with jagged edges. V shaped line.

Denticle 13 posterior side slight slope front with jagged edges. Anterior side straighter with jagged edges. W shaped line.
Denticles 14-15 posterior side slight slope. Anterior side straighter end. V shaped line.

The saw of **Sym. 608** has a total of 17 denticles. Each denticle with W or V-shaped line.
Denticle 2 posterior side slight slope. Anterior side straighter end. V shaped line.
Denticle 1 small sized. Posterior side slight slope. Anterior side slight slope with reduced jagged edges. 2 lines down.
Denticle 2 posterior side slight slope with reduced jagged edges. Anterior side slight slope with reduced jagged edges. 2 lines down.
Denticle 3 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. 2 lines down.
Denticles 4-5 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticle 6 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticles 7-10 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticle 11 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 12 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 12-14 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 15 posterior side slight slope front with jagged edges. Anterior side straighter with jagged edges. V shaped line.
Denticle 16 posterior side slight slope front with jagged edges. Anterior side straighter with jagged edges. W shaped line.
Denticle 17 posterior side slight slope. Anterior side straighter end. V shaped line.

Haplogroup 2:

The saw of **Sym. 340** has a total of 17 denticles. Each denticle with W or V-shaped line.
Denticle 1 posterior side very slight slope. Anterior side very slight slope with reduced jagged edges. 2 lines down.
Denticle 2 posterior side slight slope with reduced jagged edges. Anterior side slight slope with reduced jagged edges. W shaped line.
Denticles 3-8 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 9-12 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticles 13-15 posterior side slight slope with jagged edges. Anterior side straighter with jagged edges. V shaped line.
Denticle 16 posterior side slight slope. Anterior side straighter end. V shaped line.

The saw of **Sym. 361** has a total of 16 denticles. Each denticle with W or V-shaped line.
Denticle 1 small sized. Posterior side very slight slope. Anterior side very slight slope with reduced jagged edges. 1 lines down.

Denticles 2-7 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 8-11 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 12 posterior side slight slope with jagged edges. Anterior side straighter with jagged edges. V shaped line.
Denticle 13 posterior side slight slope with jagged edges. Anterior side straighter with jagged edges. W shaped line.
Denticles 14-15 posterior side slight slope. Anterior side straighter with jagged edges. V shaped line.

The saw of **Sym. 362** has a total of 17 denticles. Each denticle with W or V-shaped line.
Denticle 1 broad apex. Posterior side very slight slope. Anterior side very slight slope with reduced jagged edges. 2 lines down.
Denticle 2 posterior side slight slope front with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 3 posterior side slight slope front with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 4-9 posterior side slight slope front with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 19 posterior side slight slope front with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticle 11 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticle 12 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticle 13 posterior side slight slope with jagged edges. Anterior side straighter with jagged edges. V shaped line.
Denticle 14 posterior side slight slope with jagged edges. Anterior side straighter with jagged edges. W shaped line.
Denticle 15 posterior side slight slope. Anterior side straighter with jagged edges. V shaped line.
Denticle 16 posterior side slight slope. Anterior side straighter end. V shaped line.

The saw of **Sym. 589** has a total of 18 denticles. Each denticle with W or V-shaped line.
Denticle 1 very small sized. Posterior side very slight slope. Anterior side very slight slope with reduced jagged edges. 2 lines down.
Denticles 2-3 posterior side slight slope front with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticles 4-8 posterior side slight slope front with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 9-13 posterior side slight slope front with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticles 14-15 posterior side slight slope front with jagged edges. Anterior side straighter with jagged edges. V shaped line.
Denticle 16 posterior side slight slope. Anterior side straighter with jagged edges. V shaped line.
Denticle 17 posterior side slight slope. Anterior side straighter end. V shaped line.

The saw of **Sym. 602** has a total of 16 denticles. Each denticle with W or V-shaped line.
Denticle 1 posterior side slight slope. Anterior side slight slope with reduced jagged edges. 2 lines down.
Denticles 2-9 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticle 10 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. V shaped line.
Denticles 11-12 posterior side slight slope with jagged edges. Anterior side slight slope with jagged edges. W shaped line.
Denticles 13-14 posterior side slight slope with jagged edges. Anterior side straighter with jagged edges. W shaped line.
Denticle 15 posterior side slight slope. Anterior side straighter end. W shaped line.